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Departamento de Engenharia de Materiais e Cerâmica

Ana João Nóbrega

Scaffolds fibrosos e anisotrópicos para aplicação de tecidos da cartilagem

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Materiais e Dispositivos Biomédicos, realizada sob a orientação científica do Doutor António Manuel Godinho Completo, Professora Associado com Agregação do Departamento de Engenharia Mecânica da Universidade de Aveiro e da Doutora Paula Alexandrina de Aguiar Pereira Marques, Equiparada a Investigadora Principal do Departamento de Engenharia Mecânica da Universidade de Aveiro

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“It always seems impossible until it is done”

- ***Nelson Mandela***

o júri

presidente

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Professora Associado com Agregação da Universidade de Aveiro

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Doutora Paula Alexandrina de Aguiar Pereira Marques
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palavras-chave *Electrospinning, Scaffolds*, Policaprolactona, Hidrogel, Cartilagem, Engenharia de Tecidos.

resumo

O principal desafio associado à engenharia de tecidos da cartilagem é a dificuldade em recriar a organização estrutural das redes fibrosa natural do tecido cartilaginoso, com o objetivo de simular as propriedades mecânicas do tecido natural e, portanto, otimizar a resposta celular.

A importância da estrutura de colagénio para as propriedades mecânicas da cartilagem nativa é bem enfatizada na literatura. No entanto, apesar da extensa pesquisa de engenharia de tecidos, existem poucos estudos que avaliem a importância da orientação de profundidade de fibrilas de colágenio.

Durante este estudo, um *scaffold* 3D fibroso com distintas zonas biomiméticas, foi produzido, sendo posteriormente incorporado no interior de dois hidrogéis dististintos, e, seguidamente liofilizado. Os hidrogéis vão aumentar a resposta celular ao *scaffold*, enquanto que a sua liofilização vai ser responsável pelo aumento da porosidade do mesmo.

O *scaffold* foi produzido pela montagem de três zonas distintas, compostas por fibras de PCL produzidas por *electrospinning,* onde as diferentes orientações foram geradas através da variação das condições de *electrospinning* em uso.

Os resultados mostram que cada zona PCL apresenta arquitectura e topografia análogas relativamente à zona que representam no tecido nativo, originadas pela modulação precisa da orientação das fibras durante o processo de *electrospinning*.

keywords Electrospinning, Scaffolds, Polycaprolactone, Hydrogel, Cartilage, Tissue Engineering.

abstract

The major challenge associated with tissue engineered cartilage is the difficulty to recreate the organization of the natural fibrous network of the cartilaginous tissue, in order to simulate the mechanical properties of the natural tissue and, therefore, optimize cellular response.

The importance of the arcade-like collagen structure for the load-bearing properties of native cartilage is well emphasized in literature. However, despite extensive cartilage tissue engineering research; few studies have assessed the importance of collagen fibril depth orientation.

During this study, it is proposed an innovative 3D fibrous scaffold with distinct biomimetic zones, which were then incorporated into two different types of hydrogels in order to build the final structure. The produced scaffolds were later on freeze-dried, to enhance the porous network of the structure, whereas the hydrogel will increase the celluar response to the scaffold.

The scaffold was produced by the assembly of electrospun PCL fibres where the different orientations were managed by varying the electrospinning conditions in use.

Results show that that each electrospun PCL zone shows analogous architecture and topography relatively to its native counterpart due to the accurate modulation of the fibre orientation via electrospinning.

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Introduction and Objectives

Introduction and Objectives

The major challenge associated with tissue engineered cartilage is the difficulty to approximate the properties of the engineered tissues to the native ones due to cartilage's depth-dependent microstructural organization, which is divided into three functionally complementary nanofibrous zones responsible for balancing optimal mechanical properties with an enhanced biochemical cell response[1][2]. Various experimental studies in bioreactors had concluded that the mechanical properties of engineered cartilage can be improved by appropriate mechanical stimulation. These indications are promising, however, the properties of these engineered cartilages remain inferior to native. The major shortcoming of tissue-engineered cartilage is believed to be the lack of collagen content and consequently its poor tensile properties. Another limitation is that tissue-engineered cartilage does not possess native zonal variations. The importance of the arcade-like collagen structure for the load-bearing properties of native cartilage is well emphasized in literature .However, despite extensive cartilage tissue engineering research. Few studies have assessed the importance of collagen fibril depth-orientation on the mechanical properties of engineered cartilage.

The main goal of this study was the development of a three-dimensional (3D) anisotropic nanofibrous scaffold with depth-dependent variations in fibre size and orientation able to mimic the natural collagen (Col) fibrous network present on the cartilage microenvironment.

Indeed, the distinctive features of each cartilaginous zone were replicated by producing polycaprolactone (PCL) fibres using different electrospinning set ups and then assembled within a Col hydrogel, leading to an innovative 3D structure capable of combining an accurate organizational and morphological fibre distribution with a biocompatible porous system. PCL was selected to produce the fibres since it exhibits a very slow degradation rate that finds many applications in biomedical science owing to its superior mechanical properties, good biocompatibility, and complete degradation to nontoxic by-products. It is a Food and Drug Administration approved material. [3]

A Nanofibre Electrospinning machine was used and the electrospinning operating conditions were adapted in order to obtain anisotropic scaffolds. A technique to incorporate multiple electrospun layers with distinct fibre sizes and orientations was of sequential electrospinning onto different collectors in which process, the electrospinning

conditions are also different. The production of multilayer electrospun scaffold by varying both the fibre size and orientation resulted in the development of a structure with different zonal fibre orientation and tensile properties.

Several characterization techniques were used along this work to analyse the fibres and the constructs, namely SEM, FTIR analysis and mechanical testing.

This dissertation is organized in four chapters.

The first chapter is the Literature Review, where the main problem proposed for this dissertation is addressed and a short review is made of the methods used to produce this type of scaffold.

Material and Methods of the all work performed is presented in Chapter II.

Chapter III presents all the results and discussion of the work. Finally, in the last chapter, the main findings of this dissertation and references to future prospects and possible improvements of this work are presented.

Chapter I

1. State of the Art

1.1. Natural Cartilage

The cartilaginous tissue is a specialized form of connective tissue, which has a rigid consistency.[4] It functions as a soft tissue support and the coating of articular surfaces. Cartilage tissue is crucial in the development of bone tissue, specifically the formation and growth of long bones.

Depending on its composition, cartilage may be classified as elastic, fibrocartilage and hyaline or articular cartilage. Elastic cartilage maintains the shape of certain human structures. Fibrocartilage is a connective tissue with intermediate characteristics, acting as a surface coating of bone extremities. Synovial joints require a structure that avoids the direct contact between the bone surfaces, allowing a bland sliding between them. This coating structure is articular cartilage. In general, the synovial joint has a capsule that covers all structures, to provide stability to the joint surfaces. The inner face of the capsule is surrounded by a thin membrane called the synovial membrane, whose primary function is the segregation of synovial fluid which serves as a lubricant for the joint surfaces. This allows its sliding with low friction, which promotes the improvement of the mechanical behavior of cartilage.[2]

Cartilage, with the exception of articular and fibrous cartilage, is surrounded by a connective tissue layer called perichondrium. This layer follows cartilage throughout its entire surface and it possesses nerves and lymphatic and blood vessels. [4]

Such as the other types of connective tissue, cartilage is composed by cells, the chondrocytes, surrounded by the extracellular matrix. The matrix cavities are filled with chondrocytes. [4]

The cartilaginous tissue functions depend mainly on the matrix's structure, whose main component is collagen, or collagen and elastin, proteoglycan-associated molecules, hyaluronic acid and other glycoproteins. [4]

As the collagen and elastin are flexible, the main component responsible for the stiffness of the cartilage is primarily the existence of electrostatic bonds between the collagen and glycosaminoglycans, and the amount of water that is associated with these glycosaminoglycans.

The cartilaginous tissue has no blood vessels and therefore their nutrition is made from the capillaries from the surrounding tissue, designated perichondrium. The articular cartilage does not have perichondrium, their nutrition is made from the synovial fluid

present in the joint cavities. Exceptionally, the blood vessels can traverse cartilage, in order to nurture other tissues. The cartilage tissue also lacks lymph vessels or nerves .[4]

1.1.1. Cartilage Cells: Chondrocytes

The articular cartilage is composed of a small number of cells designated as chondrocytes, surrounded by an extracellular matrix, a characteristic property of all kinds of connective tissue. The chondrocytes constitute about 1-10% of the total volume of the cartilage tissue.[5]

They have the function of producing proteins of the extracellular matrix, as well as cytokines and enzymes responsible for destroying the matrix. In the cartilage tissue, the cellular mode of operation is a little different from other tissues. This is due to the lack of distinct groups of cells, able to carry out production and cell destruction functions. In the case of cartilage due to its extreme specialization there is only one cell type responsible for performing any of these functions - chondrocytes. [5] It is the balance between capacity and cell degradation that lays the cartilage physiology and function of articular cartilage. Chondrocytes are cells that secrete collagen, especially Type II, proteoglycans, and glycoproteins. [4] Because cartilage does not possess blood vessels, these are cells which are oxygen deficient. Most nutrients are transported by the blood, pass through the perichondrium and move up to the deepest chondrocytes by diffusion through the cartilage matrix.[4]

1.2. Articular Cartilage

This is the most abundant type of cartilage in the human body. It is located between the shaft and the epiphysis of long bones and, in the growth phase, it is responsible for the growth of the extent to bone.

Regarding its composition, the hyaline cartilage has about 40% of its dry weight of type II collagen fibres associated with hyaluronic acid, proteoglycans, and glycoproteins.

The high water content coming from the solvation of glycosaminoglycan molecules acts as a system that absorbs mechanical shocks, especially regarding the joint cartilage. Cartilage promotes reduced surface friction forces, and thus reduces wear on the existing joints (Figure 1). This displays anisotropic mechanical properties resulting from differences in density and structural arrangements of extracellular matrix, consisting predominantly as stated in proteoglycan molecules, together with type II collagen fibres.[6]

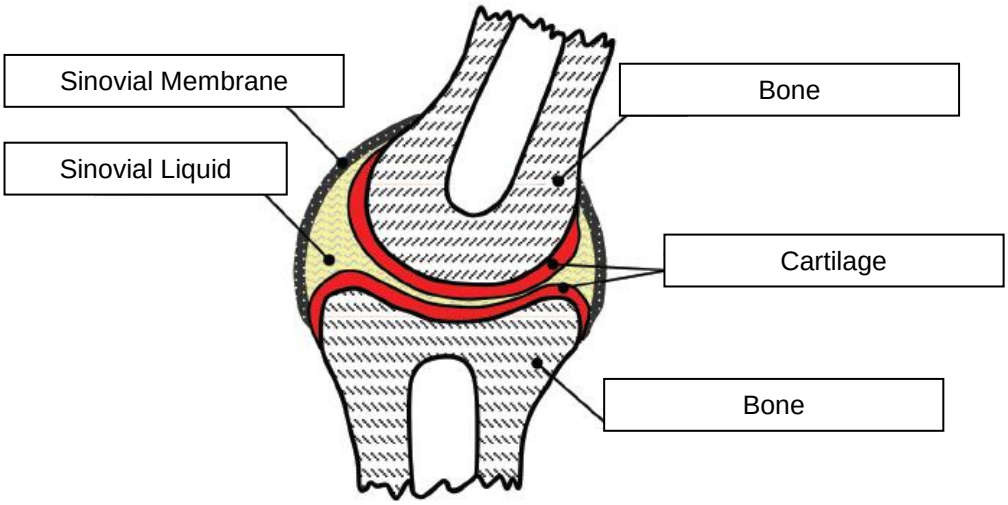


Figure 1: Schematic representation of an articular joint. Adapted from [5]

The collagen meshes enhance the mechanical properties of cartilage. According to three location areas, collagen fibres have different alignments: a superficial zone, where these fibres are aligned parallel to the region; the middle zone, where the alignment of these fibres is random and in the deepest zone, the fibres are aligned perpendicular mind the subchondral surface.

Thus, the different orientations of fibres will promote different mechanical features depending on the zones in which they are. [6]

1.2.1. Structure and composition of articular cartilage

Articular cartilage is hyaline cartilage and is 2 to 4 mm thick.[7]Besides the collagen fibre ultrastructure and ECM, chondrocytes also have an impact on the various zones of articular cartilage—the superficial zone, the middle zone, the deep zone, and the calcified zone.[8]

The superficial (tangential) zone is responsible for the protection of deeper layers from shear stresses. Its volume takes 10% to 20% of articular cartilage thickness. The collagen fibres of this zone are aligned parallel to the articular surface (Figure 2). The superficial layer contains a relatively high number of flattened chondrocytes, and the integrity of this layer is imperative in the protection and maintenance of deeper layers. This zone is in contact with synovial fluid and is responsible for most of the tensile properties of cartilage, which enable it to resist the sheer, tensile, and compressive forces imposed by articulation.[7]

Immediately deep to the superficial zone is the middle (transitional) zone, which provides an anatomic and functional bridge between the superficial and deep zones. The middle zone represents 40% to 60% of the total cartilage volume, and it contains proteoglycans and thicker collagen fibrils.[8]

In this layer, the collagen is organized obliquely, and the chondrocytes are spherical and at low density. Functionally, the middle zone is the first line of resistance to compressive forces.[8]

The deep zone is responsible for providing the greatest resistance to compressive forces, given that collagen fibrils are arranged perpendicular to the articular surface. The deep zone contains the largest diameter collagen fibrils in a radial disposition, the highest proteoglycan content, and the lowest water concentration. This zone represents approximately 30% of articular cartilage volume.[9]

The collagen fibrils are arranged perpendicular to the articular cartilage. The calcified layer plays an integral role in securing the cartilage to bone, by anchoring the collagen fibrils of the deep zone to subchondral bone. [7]

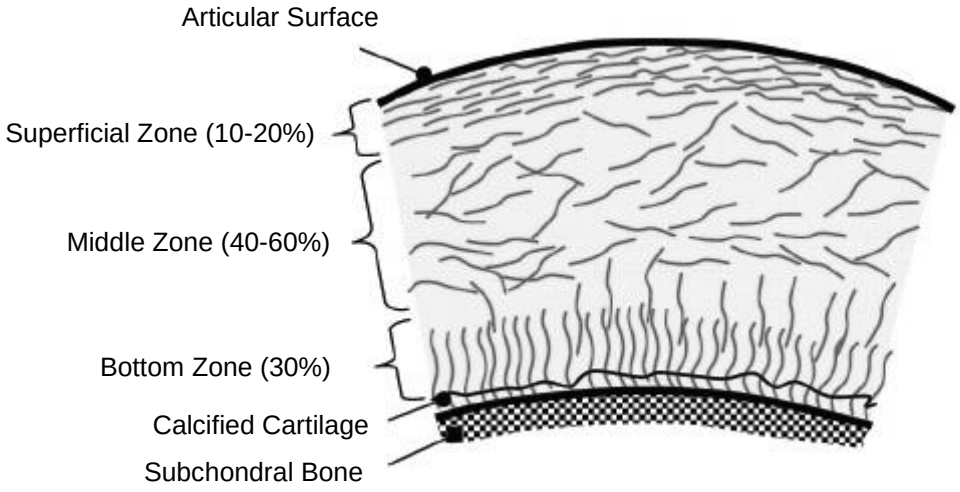


Figure 2: Cartilage's zonal organization in terms of collagen fibre orientation. Adapted from [5]

1.3. Tissue engineering for cartilage repair

Tissue engineering promotes the use of methods which boost cell growth, through the manipulation of a wide range of biomaterials, and these can be either natural or not. These biomaterials will be required to support different types of human tissues. These biomaterials will be required to support different types of human tissues [10]

Through the last twenty years there has been a huge expansion in the field of biomaterial technologies, cell sources and molecular and genetic manipulations that could positively impact the development of a truly functional tissue engineered cartilage substitute. [11]

To date, tissue-engineering cartilage strategies have had some success, developing replacement tissue constructs with biochemical properties approaching that of native cartilage.[11]

From a biomechanical point of view, cartilage tissue functionality is defined by parameters like permeability and stiffness, which can be related to tissue components such as glycosaminoglycan, collagen content and its structural organization. [2]

Strategies for regenerating tissue used in tissue engineering, consist on the use of living cells as an engineering material. The manipulation of these cells involves a number of different specialized tools and processes among which are the cell culture scaffolds.[2]

In recent years, progress in health, combined with engineering and other branches of science have allowed a great evolution of reconstruction techniques of total or partial functions of organs and tissues. In this context, the biomaterials have gained great importance, which is associated with the development of new surgical techniques and implants and prosthetics. [12] These biomaterials have been used in a wide range of structures that enable cell growth and proliferation on damaged body tissues.

Such structures are called scaffolds, and they are built with materials whose mechanical, physical and chemical properties are ideal for the adhesion and proliferation of cells and consequent formation of new tissue.

These phenomena can occur both in vivo or in vitro, in which the first relates to the implantation of the scaffold in the place where it is desired, in order to correct an existing defect or injury in a patient's tissue. In the second case, the tissue cultivation is done in controlled environments, whose characteristics promote cell growth. After the cell growth, the already formed tissue is implanted in the patient. [13]

Despite the fact that the goal of tissue engineering is essentially clinical, there is also a great potential for use in pathology and pharmacology, meaning that recreated tissues with these techniques, which are very similar to the original tissue, can be used to test drugs under investigation and study the behavior of diseases, without any risk to human health.[14]

To date, tissue-engineering cartilage strategies have had some success, developing replacement tissue constructs with biochemical properties approaching that of native cartilage. However, poor biomechanical properties and limited post-implantation integration with surrounding tissue are major shortcomings that need to be addressed.[10]

Articular cartilage lesions are a particular challenge for regenerative medicine strategies, due to the fact that cartilage’s function stems from its complex depth-dependent microstructural organization, mechanical properties, and biochemical

composition. Repairing such lesions with engineered scaffolds containing live cells remains an important aim in the field of regenerative medicine. [2]

As it was referred, articular cartilage exhibits anisotropic mechanical properties as a result of depth dependent differences in the density and structural arrangement of its extracellular matrix, which consists predominantly of proteoglycan molecules retained within a fibrillar type II collagen meshwork. The fibrillar collagen meshwork provides mechanical reinforcement and is comprised of three zones, namely the superficial, middle, and deep zones.[2]

A number of strategies have been explored for articular cartilage repair, including the use of synthetic polymer fleeces, sponges, and fibrous materials that can be seeded with the patient's own chondrocytes or stem cells and either sutured or packed into defect sites.[2]

The field of cartilage tissue engineering has been developing over the last twenty years but, despite extensive efforts to develop novel biological solutions, there is still a lack of clinical options for treatment. Although the field has concentrated on finding therapies for focal lesions, it has now developed sufficiently to begin considering the challenge of finding new solutions for the extensive joint damage seen in osteoarthritis.[15]

The last twenty years have been witness to a huge expansion in the field of biomaterial technologies, cell sources and molecular and genetic manipulations that could have positive impact on the development of a truly functional tissue engineered cartilage substitute.[16]

Biodegradable scaffolds that are functionally graded in terms of organization, porosity, pore size, and mechanical properties may provide distinct advantages over scaffolds that feature uniform properties and homogeneous compositions. Specifically, scaffolds with depth-dependent variations in the fibrillar size and orientation that mimic the composition and structure of the native tissue may offer a superior template for cellular organization and extracellular matrix production. [14]

1.3.1. Important parameters for tissue engineering studies

1.3.1.1. Cell Source

The ideal cell source for cartilage tissue engineering is the one which is able to be easily isolated and expanded, and which synthesizes abundant cartilage-specific extra-cellular matrix components. [14]

Chondrocytes are the most obvious cell source. They are able to produce, maintain and remodel the cartilage in vitro. Nevertheless, only a small number of autologous chondrocytes are available, and cells harvested from diseased joints are relatively inactive. Unfortunately, chondrocytes expansion in monolayer causes dedifferentiation, characterized by decreased proteoglycan synthesis and type II collagen expression and increased type I collagen expression.[14]

Chondrocytes' age is also a subject in need of consideration. Most cartilage tissue engineering studies, chondrocytes from immature animals are used, which proliferate faster and have increased chondrogenic potential compared to chondrocytes from older human donors.[14]

1.3.1.2. Types of scaffolds

The goal of the use of biomaterial scaffolds in cartilage tissue engineering is to provide the cells with a comfortable niche which stimulates cells to synthesize cartilage matrix, and to (temporarily) replace the function of the native matrix until new cartilage has formed. To fulfill that function, the scaffold should preferably:

a) Possess adequate mechanical properties, that is the scaffold should have sufficient mechanical strength to withstand the pressures on the environment where it is deployed;[17] [18]

b) Their degradation rate should be adjusted, taking into account the rate of tissue regeneration; [17]

c) Porosity, size and structure of pores are factors of great importance in the transport of nutrients and cell proliferation which means that the size and distribution of the pores should be sufficient to promote cell growth without altering the mechanical properties of the scaffold. [17][18]

d) The scaffold must have suitable surface chemical properties in cell adhesion, combined with a sufficient roughness, taking into account that the adherence is a prerequisite for future cellular functions. The different types of cells require different substrates to achieve different types proliferate and promote differentiation. Once the surface features, topography, chemical properties and its humidification have a key role in

cell adhesion and is common to receiving scaffolds surface treatments to improve this; [17][18]

e) Prior to its implementation, the scaffold will have to be sterilized. Thus, the materials used for construction must be ideal to survive sterilization, either by exposure to high temperatures, vapour of ethylene oxide, or gamma radiation and should remain unchanged when subjected to these techniques. [18]

f) Finally, the scaffold should be easy production and processing, any kind of three-dimensional geometry, since these are often used in defects only, irregular and complex shapes, so the scaffold must be easy and versatile manufacturing. [18]

Many natural and synthetic polymers have been used as scaffold material in cartilage tissue engineering. [14]

1.3.1.3. Materials in use

The choice of a suitable material is a crucial step in the development of scaffolds for tissue engineering applications since the selected material will be responsible for enabling the generation of a useful size and volume of tissue, allow an efficient delivery of molecular and mechanical signals to the cells and it should also support the cells and optimize their function within the scaffold. In fact, as the goal of the scaffold is to mimic the advantageous characteristics of the natural extracellular matrix (ECM), the scaffolding material should present physical, chemical and biological properties that enhance biocompatibility and biodegradability in order to avoid unwanted host tissue reaction of the immune system and the damage of the tissues via toxic products, respectively.[19]

Some of the most common categories of biomaterials used in the fabrication of scaffolds for tissue engineering are ceramics, natural and synthetic polymers, since these kinds of materials offer design flexibility due to its composition, structure and arrangement of their constituent macromolecules can be adapted to different functions. Generally, polymers present appropriate properties for tissue engineering applications like biocompatibility, high surface-to-volume ratio, high porosity, biodegradation and good mechanical features. [20]

The natural polymers have a very elevated potential for achieving clinical success because they normally exhibit high levels of biocompatibility, biodegradability and low immunogenicity, which consequently enhance the cells performance in the biological systems. [19] Many of these are hydrogels, which makes them appropriate for engineering tissues such as cartilage, which have high water content. Hydrogels are interesting for studies in which mechanical loading is used, because they are able to transduce

mechanical loads such that forces can be exerted on the cells. [21] Finally, natural scaffold materials allow natural ECM remodeling. [21]

The most widely used synthetic polymeric scaffolds in cartilage tissue engineering are the poly-α-hydroxy esters, especially polylactic acid (PLA), polyglycolic acid (PGA), and Polycaprolactone, because of their biodegradability and US Food and Drug Administration (FDA) approval for clinical use. [14] Scaffolds composed by these polymers have better mechanical strength than hydrogels, which makes it easier to fix them in a defect and improves their load-bearing properties. [14]

A disadvantage of synthetic polymers is that cells often do not maintain their chondrocytic phenotype and produce ECM with inferior properties. [14]

1.3.1.4. Scaffold architecture, porosity and stiffness

Porosity, pore size and interconnectivity of scaffold materials are important since these properties influence cell migration and diffusion of oxygen, nutrients, waste products and signaling molecules. [14]

In addition, a porous material improves mechanical stability at the interface. Porosity and permeability have a remarkable effect on proliferation and phenotype of chondrocytes.

Stiffness of scaffolds also influences the mechanical environment of the seeded cells which in turn can influence cell differentiation and tissue growth in culture [14].

Supplementary, the load on cartilage is a stress and not a strain, hence the strain applied to the cells at first is a function of the scaffold stiffness and then a combination of scaffold and ECM properties as the tissue is produced. [14]

1.3.1.5. Biodegradability

Spatial and temporal controlled degradation of the scaffold can affect production and deposition of new tissue. Therefore, an optimal degradation kinetics ensures initial stability and shape of the scaffold.[14]

Several degradable scaffolds have been adopted for cartilage tissue engineering. It has been shown that scaffolds that degrade slowly lead to increase and more homogeneous ECM deposition when compared to fast degrading scaffolds [14]. Also, the degradation of the scaffold allows for integration and remodeling of the new tissue into the surrounding cartilage after implantation. [15]

1.4. Polycaprolactone

Polycaprolactone (PCL) was one of the earliest polymers synthesized by the Carothers group in the early 1930s. [3] It became commercially available following efforts to identify synthetic polymers that could be degraded by microorganisms. PCL can be prepared by either ring opening polymerization of ϵ-caprolactone using a variety of anionic, cationic and co-ordination catalysts or via free radical ring-opening polymerization of 2-methylene-1- 3-dioxepane.[3]

Recently, PCL has gained a lot of attention, and shown great potential in biomedical applications. As it was previously mentioned, among synthetic polymers, PCL is one of the easiest to process and manipulate into a large range of shapes and sizes due to its low melting temperature and its superior viscoelastic properties.

PCL is a hydrophobic, semi-crystalline polymer, more specifically a polyesther whose crystallinity tends to decrease with increasing molecular weight. Its good solubility, low melting point (59–64 ^{o}C) and exceptional blend-compatibility has stimulated extensive research into its potential application in the biomedical field. [22]

PCL and its copolymers have been used in an extended number of drug-delivery devices. Attention was drawn to these biopolymers owing to their numerous advantages over others in use. Functional groups could also be added to render the polymer more hydrophilic, adhesive, or biocompatible which enabled favorable cell responses. [3]

There is a wide range of applications for PCL, such as scaffold fabrication for tissue engineering applications, more specifically bone, cartilage, cardiovascular skin and nervous tissue engineering, due to its relatively inexpensive production routes, compared with other aliphatic polyesters. Furthermore, the fact that a number of drug-delivery devices fabricated with PCL already have FDA approval and (CE) Mark registration enables a faster avenue to market.[3]

This polyesther is soluble in chloroform, dichloromethane, carbon tetrachloride, benzene, toluene, cyclohexanone and 2-nitropropane at room temperature.[22]

PCL is not degraded by enzymes within human or animal bodies because of the lack of suitable enzymes, but it can be degraded by microorganisms (bacteria and fungi) as the polymer chains have ester linkages that are hydrolysable. The hydrolysis can also occur under in vitro and in vivo conditions, although the rate of degradation is slow.[22]

1.4.1. Cartilage tissue engineering using PCL

As said in section 1.2. of chapter I, cartilage is an elastic, avascular tissue composed of chondrocytes entrapped in an ECM rich in proteoglycans and collagens. [6] Cartilage has low self-renewal ability, due to the absence of vascular networks or progenitor cells within the tissue. [22] A recent study demonstrated the dissimilar chondrogenic differentiation behaviour of mesenchimal stem cells which derived from different tissues. These cells were seeded onto oriented nano and microfibrous electrospun PCL scaffolds as well as a random porous PCL film were shown to achieve a structure similar to articular cartilage. Cell viability and cell morphology on the fibrous scaffolds showed that engineering an oriented ECM environment to regulate tissue alignment could be optimized by oriented electrospun nanofibres. Recently, electrospun PCL and starch-combined PCL nanofibre webs were used by researchers to evaluate ECM formation by bovine articular chondrocytes. [3]

More recently, researchers have fabricated 3D cell-printed scaffolds using layer-by-layer (LBL) deposition of PCL and chondrocytes encapsulated within alginate hydrogels using additive manufacturing methods. [3]

1.5. Graphene Oxide

Graphene is a single layer of aromatic carbon atoms in a two dimensional lattice, whose derivatives have attracted great attention in the preparation of high-performance and functional nanofibre scaffolds because of their unique physicochemical, electrical, thermal, and biological properties [23]. They have been widely used in the fields of energy, electronics, and biomedicine. This material can either be obtained by a bottom-up approach via chemical vapor deposition or a top-down strategy like mechanical exfoliation of graphite. The latter is an indirect route involving chemical exfoliation of graphite to graphene oxide (GO) followed by reduction is common due to high yield and low-cost.[23]

GO presents a layered structure which is similar to graphene. However, its carbon sheets are oxygenated with hydroxyl and epoxy functional groups on the basal plane and carbonyl/carboxylic acids groups on the plane edges which are responsible for making GO's surface highly hydrophilic, leading to the formation of stable aqueous colloids.[24]

GO offers the possibility of combining oxygen-containing groups with specifc functional groups of biomolecules or other polymers. This can be via either covalent or non-covalent methods in order to improve this material.[24]

Recently, GO has received much interest in the field of regenerative medicine and studies have shown that it can positively influence the attachment, proliferation and differentiation of stem cells.[25]

GO has been shown to enhance the bulk properties of materials such polycaprolactone, giving place for GO composites that are able to successfully mimic biological environments.[23]

Cross-linked hydrogels, composed by GO are particularly attractive in the biomedical field, mainly because they offer the ability to mimic living-tissue. [25]

1.6. Chitosan

Polysacharides are widely distributed in nature. Among them, Chitosan (Chit) is receiving increasing attention. Chit is a linear polysaccharide prepared from chitin, which is the second most abundant natural polymer in the world. Chitin can be found in the exoskeleton of insects, crustaceans and some cell wall of fungi. This polymer can be obtained by alkaline deacetylation or enzymatic hydrolysis. [26]

Chit is a polymer of great interest in pharmaceutical, medical and biomedical fields, for the latter, more specifically in tissue engineering due to its biological activities such as non-toxicity, antimicrobial activity, biocompatibility, biodegradability.[27] These properties improve the therapeutic effects making Chit a polymer with a great potential for biomedical and biopharmaceutical applications, especially for drug delivery.[27]

The presence of NH2 groups in Chit are responsible for a much higher potential, when in comparison with chitin.[26] This makes it an excellent polymer for pharmaceutical applications. [28]

The properties of Chit mentioned above make it an exciting and promising for tissue engineering purposes, with a large margin for development.[27]

1.7. Collagen

As it was mentioned in section 1.2. of chapter I, collagen is the principal extracellular matrix component for many tissues and the most commonly used biomaterial in regenerative medicine applications. It is one of the main components of cartilage tissue. [4]

Collagen is widely used in tissue engineering since it can easily form a hydrogel structure at physiological pH capable of simulating the ECM.[25] Additionally, this polymer can easily and successfully be integrated in hybrid networks with other materials,

including GO, in order to enhance the biocompatibility and biodegradability of the system.[25]

1.8. Electrospinning

Electrospinning is a technique which has been used for the development of tissue engineering structures. The equipment is composed by a high voltage power supply, which creates an electric field between a grounded collector and a positively charged capillary, which contains a polymer solution. When the applied voltage is sufficiently high, the droplet in the capillary, the body of the liquid becomes charged, and when the electrostatic charge overcomes the superficial tension of the polymer solution in the capillary tip, a polymer jet is created. [28]

As the jet dries, it elongates due to a whipping process created by electrostatic repulsion initiated at small bends on the fibre, until it hits the collector. The elongation and thinning of the fibre allows its removal from the collector with various orientations in order to develop different structures with unique compositions and mechanical properties. [28]

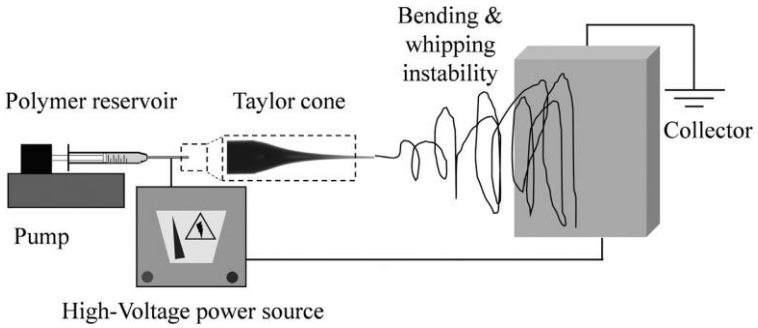


Figure 3: Electrospinning's set-up schematically represented[28]

Electrospinning generates loosely connected 3D porous mats with high porosity and therefore high surface area that can mimic the ECM, which makes 3D scaffolds developed by this technique eligible for tissue engineering applications. [28]

It is evident; from the analysis of vast literature that electrospinning of any biodegradable and biocompatible polymer is no longer a problem. By adjusting the electrospinning conditions like voltage, distance to the collector, flow rate of the solution during the technique and solution properties such as viscosity, conductivity, any polymer can be electrospun.[29]

In the current days, mimicking ECM by electrospinning a wide variety of materials is easily achievable. The efficiency of using the process for any tissue engineering application can be further determined by the incorporation of the bioactive groups into the

structures. This incorporation could lead to the biofunctionalization of engineered scaffolds due to the fibres' biofuctionalization as well, which will have an impact on the efficiency of these fibres for the regeneration of biological tissues [29]

1.8.1. Electrospinning conditions

1.8.1.1. Voltage

It is a known fact that the flow of current from a high-voltage power supply into a solution via a metallic needle will cause a spherical droplet to deform into a Taylor cone and form ultrafine nanofibres at a critical voltage which is determined by the type of polymer. The formation of smaller-diameter nanofibres with an increase in the applied voltage is attributed to the stretching of the polymer solution in correlation with the charge repulsion within the polymer jet. This said, it is expectable that, the higher the voltage, the lower the fibre diameters. [30]

1.8.1.2. Flow rate

The flow of the polymeric solution through the metallic needle tip determines the morphology of the electrospun nanofibres. [30]

Increasing the flow rate beyond a critical value not only leads to increase in the pore size and fibre diameter but also to bead formation (due to incomplete drying of the nanofibre jet during the flight between the needle tip and metallic collector) .Because increases and decreases in the flow rate affect the nanofibre formation and diameter, a minimum flow rate is preferred to maintain a balance between the leaving polymeric solution and replacement of that solution with a new one during jet formation.[30]

Any change in the surface charge density may also affect the morphology of the nanofibre. For instance, Theron et al. revealed that the flow rate and electric current are directly related to each other.[31]

1.8.1.3. Effect of needle to collector distance and needle diameter

The distance between the metallic needle tip and collector plays an essential role in the determination of the morphology of an electrospun nanofibre. Similar to the applied electric field, viscosity, and flow rate, the distance between the metallic needle tip and collector also varies with the polymer system in use. The nanofibre morphology could be easily affected by the distance because it depends on the deposition time, evaporation rate, and whipping or instability interval. [30]

A critical distance needs to be maintained to prepare smooth and uniform electrospun nanofibres. [30]

1.5.1.4. Effects of polymer concentration and solution viscosity

The electrospinning process relies on the phenomenon of the uniaxial stretching of a charged jet. The stretching of the charged jet is significantly affected by changing the concentration of the polymeric solution. For example when the concentration of the polymeric solution is low, the applied electric field and surface tension cause the entangled polymer chains to break into fragments before reaching the collector. [30] These fragments cause the formation of beads or beaded nanofibres. Increasing the concentration of the polymeric solution will lead to an increase in the viscosity, which then increases the chain entanglement among the polymer chains. These chain entanglements overcome the surface tension and ultimately result in uniform beadless electrospun nanofibres. [30]

1.9. Electrospinning techniques for cartilage tissue engineering

1.9.1. Electrospinning of PCL solutions for the development of 3D structures

A number of strategies have been explored for articular cartilage repair, including the use of synthetic polymer sponges, and fibrous materials that can be seeded with a patient's own chondrocytes or stem cells and either sutured or packed into defect sites.[32]

Although these therapies can are able to boost neocartilagenous tissue formation, in vivo, their homogenous design likely limits successful outcomes. The newly formed tissue tends to suffer marginal integration in the surrounding cartilaginous tissue and poor mechanical properties due to the production of fibrocartilage, both of which may be because of the lack of similarities in structure and organization within such scaffolds, when in comparison with the native tissue. Therefore, in an effort to mimic the fibrillar network of articular cartilage, electrospun scaffolds comprised of fibres with diameters on the micron-to-submicron size scale have also been explored, as these materials are able to mimic natural cell– extracellular matrix interactions, promote production of cartilage-like tissue, and provide a template to organize the newly deposited matrix.[33]

Electrospinning is an easily reproducible echnique capable of generating dense, compliant, biocompatible fibre networks with a wide range of physical properties such as fibre size, spacing, orientation, and tensile properties.[34]

An interesting technique to incorporate multiple electrospun layers with distinct fibre sizes and orientations is one in which several different electrospinning conditions are applied, using the same collector. This method has been employed to create bilaminar, trilaminar, and multilayer scaffolds to repair blood vessels. For cartilage tissue regeneration, the studies are not very abundant. [2]

However, Mc Cullen et al. tried to develop a trilaminar scaffold by varying both the fibre size and orientation to create scaffolds that mimic both the fibre orientation and the zonal tensile properties of articular cartilage. Trilaminar composite electrospun scaffolds were directly compared with homogenous scaffolds formed from either randomly oriented or aligned fibres in terms of their compressive mechanical properties and the ability to support in vitro cartilage formation. [2] Results showed that, by the comparison of these trilaminar scaffolds to homogeneous scaffolds, the scaffolds composed by different layers are more suitable for cartilage regeneration techniques.

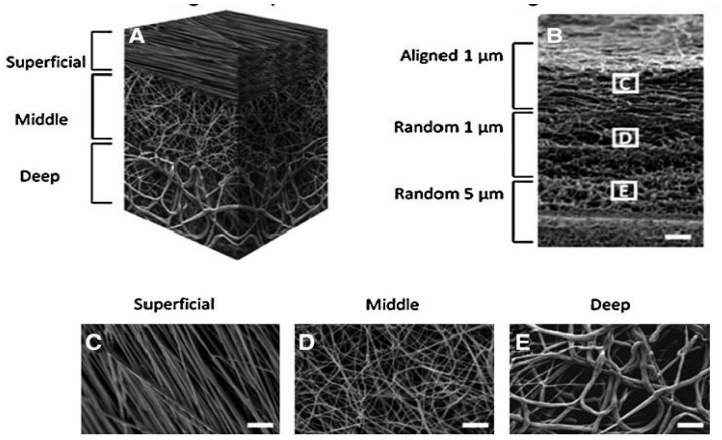


Figure 4 Schematical representation vs actual results of Steele et al. research. The diagram (A) illustrates the zonal differences, resulting from the variation of the electrospinning conditions. images of the bulk anisotropic scaffold (B) and the varying fibres of aligned (C), randomly oriented (D), or randomly fibres throughout the scaffold (E). [2]

Steele et al. also tried to generate a bilayered poly(e-caprolactone) (PCL) scaffold comprised of an aligned fibre zone, which mimics the morphology and mechanics of the superficial zone of articular cartilage, laminated to a bulk porous particulate-templated scaffold which allows for full cellular infiltration and extensive ECM deposition. It was hypothesized that the addition of aligned electrospun fibres would increase tensile

properties, reduce surface roughness, and provide morphological similarities to native articular cartilage.[1]

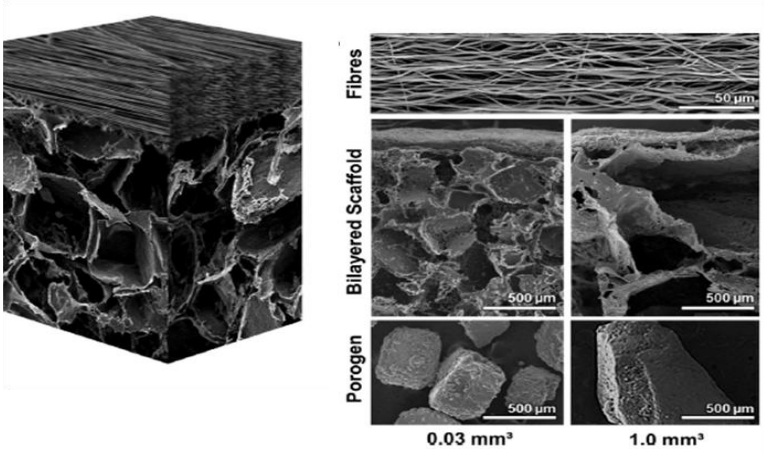


Figure 5: Bilayered cartilage scaffold schematic. (A) A diagram illustrating the electrospun fibre zone (FZ) deposited on particulate-templated foam (PZ). [1]

Both techniques presented similarities to the natural cartilaginous structure. However, they were not able to obtain full alignment for the fibers in the deep zone of the scaffold, due to the use of different approaches.

1.9.2. Wet-Electrospinning technique

Wet electrospinning is an additional option to the recurrent electrospinning technique. It was firstly introduced as a method of producing nanofibrous scaffolds for tissue engineering applications. The main difference between this and the conventional method is based on the use of a liquid collector instead of a solid, mostly metallic one.[35]

This allows the production of a bulky and fluffy material using the common electrospinning. This task could be achieved either using special collectors [29] or by the usage of porogen particles, that is, chemical blowing agents, imbedded in between the nanofibres [29]. Wet electrospinning is a relatively simple and effective method to produce 3D materials without sophisticated devices and without special chemical additives.

Hem R. et all, also tried to develop a three-dimensional scaffold via the wet-electrospinning technique, by using a water bath as a collector.[36]

1.9.3. Hydrogels

Hydrogels are usually characterized as networks of polymer chains that can be found as colloidal gels in which water is the dispersion medium. They can have the ability to swell and retain a significant fraction of water within its structure, but will not dissolve in water. Hydrogels also possess a degree of flexibility very similar to natural tissue due to their large water content. [37] This ability to absorb water is due to hydrophilic functional groups that are attached to the polymeric backbone, while their resistance to dissolution arises from cross-links between network chains. Many materials, both naturally occurring and synthetic, fit the definition of hydrogels.[37]

The synthesis of hydrogels can be performed in a number of chemical ways that include one-step procedures like polymerization and parallel cross-linking of multifunctional monomers, or multiple step procedures involving synthesis of polymer molecules having reactive groups and their subsequent cross-linking. It is possible to design and synthesize polymer networks with molecular-scale control over structure such as cross-linking density and with tailored properties, such as biodegradation, mechanical strength, and chemical and biological response to stimuli. [38]

Proteins such as collagen and gelatine and polysaccharides can have the ability to form hydrogels, Synthetic polymers that form hydrogels are traditionally prepared using chemical polymerization methods. [37] This is one easily achievable way of producing scaffolds to whom the cellular response is enhanced, due to the use of natural polymers such as the ones described, for the development of tissue engineering structures.

Chapter II

2. Development and characterization of a three layered scaffold

In this chapter, a description of the materials and methods used for the development and characterization of the scaffolds is presented.

Taking into account the promising results obtained by 3D hydrogel-fibrous scaffolds in several tissue engineering applications,[37][39] as well as the studies developed by Steel et al. [1] and Mc Cullen et al.[2] regarding the *in vitro* recreation of the cartilage native tissue, a tri-layered fibrous scaffold, in which each layer was developed by a specific electrospinning technique, was created. Each layer was then assembled inside a freeze-dried hydrogel in order to surround the eletrospun fibres in a 3D porous and connected structure for cartilage tissue engineering.

2.1. Materials

The polymer used in this study, for the electrospinning technique was Polycaprolactone (PCL) with a molecular weight of 80 KDa purchased from Sigma Aldrich®. Briefly, PCL was dissolved in two solutions of Dimethylformamide (DMF) and Dichlomethane (DCM) with a ratio of 1:1 (v:v), with concentrations of 12 and 15%, depending on the cartilaginous zone simulated. Both solvents were purchased from Sigma Aldrich®.

2.2. Electrospinning Technique

As it was mentioned on chapter two, one of the biggest challenges on this field of cartilage tissue engineering is to mimic its ECM because cartilage is divided into three complementary nanofibrous zones, responsible for balancing the optimal mechanical properties with an enhanced biochemical cell response. Indeed, since each zone exhibits distinct mechanical properties, due to fibre size and orientation, two different electrospinning techniques were applied, in order to develop two layers of aligned fibres, corresponding to the superficial and deep layers and a randomly aligned fibrous layer capable of mimicking the middle zone.

In this way, the final scaffold should suit the morphological and topographical requisites of cartilage's structural and biomechanical organization that progresses from

perpendicular to the subchondral bone surface in the deepest zone, randomly aligned in the middle zone and parallel to the articular surface on the superficial zone (Figure 2).

2.2.1. Wet Electrospinning

This technique was used with the goal of obtaining randomly aligned fibres similar to the electrospun scaffolds fabricated by Kostakova et al. [40].

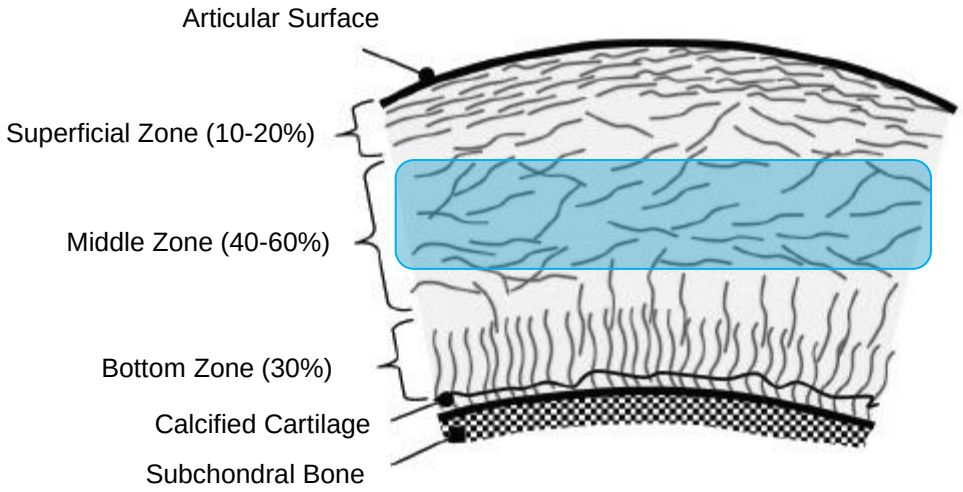


Figure 6: Schematical representation on cartilaginous tissue of the zones being created in the described electrospinning technique. Adapted from [5]

A 12% (w/v) solution was prepared by dissolving PCL in a mixture of DCM:DMF (1:1). Prior to its use, the solution was left to stir for a minimum period of 12 hours at room temperature.

Then, as it is noticeable on figure 7, a syringe containing the described solution was pumped at a controlled flow rate (1 ml/h) trough a tubing that was used to connect the syringe tip (20G) and the electrospinning nozzle to which a high voltage was applied in a range between 22 and 30 kV. The fibres were electrospun, during a period of 2 minutes and 30 seconds, into a Water (H_2O) and Ethanol (C_2H_6O) solution with a ratio of 1:9 (v:v). This liquid collector was homogenized by stirring the solution for 20 minutes before use and then placed at 4cm of the spinneret.

The process occurred at room temperature and humidity between 50 and 60%.

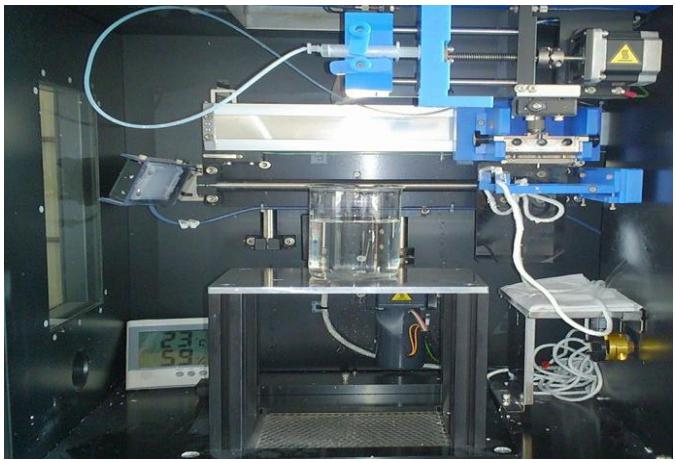


Figure 7: Wet-electrospinning set-up.

2.2.2. Dry Electrospinning

In order to obtain fully aligned fibres, a rotating mandrel was used as the collector during the electrospinning technique.

Similarly to the process described on section 2.2.1 of Chapter II, a 15% (w/v) solution was prepared by dissolving PCL in a mixture of DCM:DMF (1:1). Prior to its use, the solution was left to stir for a minimum period of 12 hours at room temperature. Then, as it is noticeable on figure 9, a syringe containing the described solution was pumped at a controlled flow rate (2.5 ml/h) trough a tubing that was used to connect the syringe tip (20 G) and the electrospinning nozzle to which a high voltage was applied in a range between 18 and 25 kV.

The fibres were electrospun, during 8 hours on an aluminum foil placed onto a rotating drum (with a diameter of 20 cm) at a working distance of 15 cm and with a velocity of 1500 rpm.

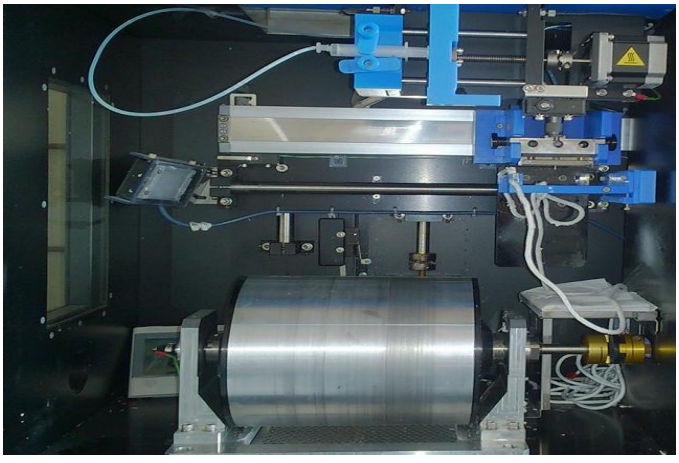


Figure 8: Electrospinning set-up.

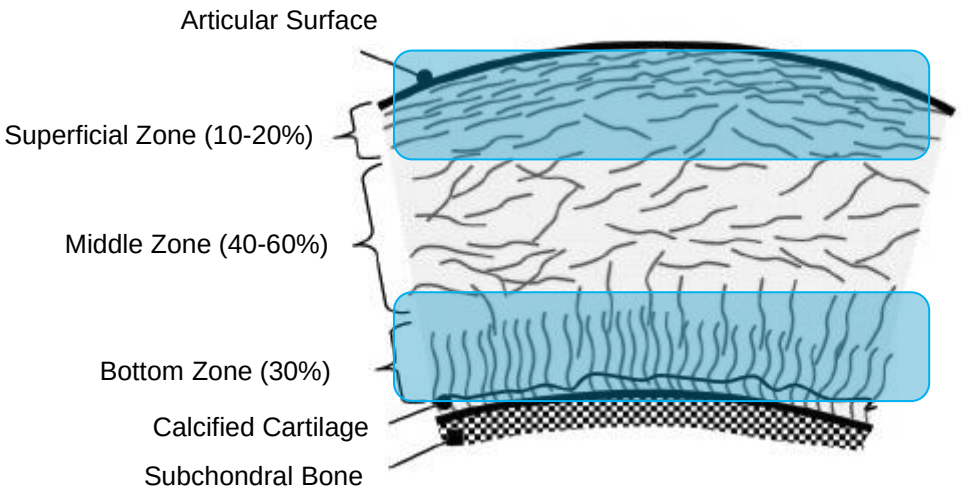


Figure 9: Schematical representation on cartilagenious tissue of the zones being created in the described electrospinning technique. Adapted from [5]

2.3. Scaffold Assembly

In order to obtain a 3D fibrous structure, it was necessary to assemble the different eletrospun layers accordingly to cartilage's morphological and topographical organization. Therefore, each layer was adapted to match the characteristics of the zone it would mimic (Figure 3). In this work, the three electrospun layers were developed with the goal of fabricating a final 3D fibrous structure with a total height of 5 mm after their assembly.

Because each layer of the scaffold was created individually (Sections 2.3.1., 2.3.2., 2.3.3.), it was necessary to find a way of setting up the final scaffold without any step backs on its main structure and function. Therefore, in order to obtain a three layered scaffold in which all of the layers are connected, the final fibrous structure was assembled inside a hydrogel, which should not only improve its biological features, but also work as a physical support for the electrospun layers, avoiding their separation. [37]

For this purpose, two kinds of hydrogels were tested: one Col hydrogel (Section 2.3.4.), and a GO-Chit-Col hydrogel (Section 2.3.5.) that after lyophilisation (Section 2.3.6.) originated a Col scaffold and a GO-Chit-Col scaffold respectively.

2.3.1. Superficial zone

The superficial zone was conceived by simply cutting the PCL electrospun sheet that was removed from the aluminum foil, as a result of the dry electrospinning process,

into small circles with 5mm diameter (Figure 10). In this way, the dimensions of the fabricated superficial zone matched the diameter of the other two zones.

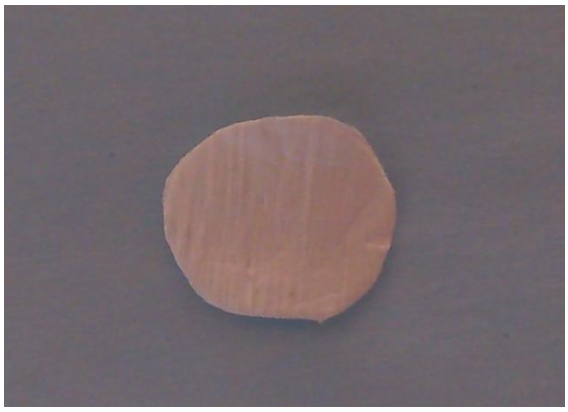


Figure 10: Final aspect of the layer representative of the top zone of the scaffold.

2.3.2. Middle zone

The middle zone was fabricated by casting the randomly aligned PCL eletrospun fibres collected from the Ethanol-Water bath used in the wet electrospinning technique, into a cylindrical mold with 5 mm diameter and 3 mm high.

Figure 11: Final aspect of the layer representative of the middle zone of the scaffold.

2.3.3. Deep zone

The deep zone was developed by cutting 3 mm high and 5 cm length rectangles from the same PCL electrospun sheet used for the development of the top zone. These rectangular electrospun sheets were rolled into a spiraled cylinder, as it is described on the Figure 12, in order to obtain a structure in which not only the fibres were vertically aligned but also a structure with a sufficient diameter (5 mm) to support the zones that would later be assembled on top of this structure.

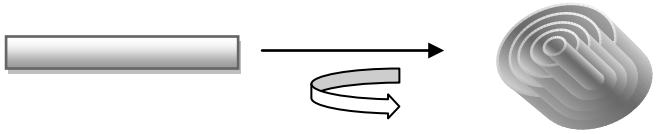


Figure 12: Scheme describing the procedure that led to the creation of the deep zone.

Figure 13: Final aspect of the layer representative of the deep zone of the scaffold.

2.3.4. Assembly of the PCL multi-layered structure into a Collagen Hydrogel

The Col hydrogel was prepared, according to literature [39], by neutralizing each 1 mL of a rat tail type I collagen solution (2.16 mg/mL protein in 0.6% Acetic Acid from First Link Ltd.) with 0.1 mL
of Dulbecco's Modified Eagle's Medium (DMEM) purchased from Sigma Aldrich® and a solution of NaOH (0.1M).

The process of scaffold assembly consisted on filling a Teflon mold (cylindrical shape with 1 cm diameter and 1 cm high) with each PCL electrospun fibrous layer, and then sequentially surrounding them with the neutralized collagen solution. As a final step, the 3D hydrogel-fibrous structure was incubated for 30 minutes at 37 ºC.

2.3.5. Assembly of the PCL multi-layered structure into a Graphene Oxide-Chitosan-Colagen Hydrogel

The GO-Chit-Col hydrogel was prepared accordingly to Girão et al.[25] Briefly, a GO aqueous dispersion (4mg/mL, water dispersion: Graphenea) was directly mixed with rat tail type I collagen solution (2.16 mg/mL protein in 0.6% Acetic Acid from First Link Ltd.) and a chitosan solution (2 mg/mL in 0.6% Acetic Acid; Chitosan was purchased from Sigma-Aldrich®) with a GO:Col:Chit w:w:w ratio of 8:1:1 and rapidly shaken for 10 seconds.

The process of scaffold assembly was the same as described in section 2.3.4 of Chapter II.

2.3.6. Lyophilisation Process

Lyophilisation or freeze drying is a process in which water is frozen, followed by its removal from the sample, initially by sublimation (primary drying) and then by desorption (secondary drying). The main principle involved in freeze drying is a phenomenon called sublimation, where water passes directly from solid state (ice) to the vapour state without passing through the liquid state.[41]

In this case, the freeze-drying process would produce a biocompatible porous network around the PCL electrospun fibrous structure, promoting cell adhesion and proliferation.[25]

Therefore, the final assembled scaffolds (Col scaffold and GO-Chit-Col scaffold) were obtained after freeze-drying the 3D hydrogel-fibrous structures via a lyophilization process (Teslar IoQuest HT40, Beijer Electronics Products, and Malmoe, Sweden) at -80 ˚C.

Both the Col and GO-Chit-Col scaffolds present specific coloring. The Col scaffold (Figure 14 (a)) presents a pink color due to the use of DMEM, whereas Graphene Oxide is responsible for the GO-Chit-Col scaffold's (Figure 14 (b)) brown color.

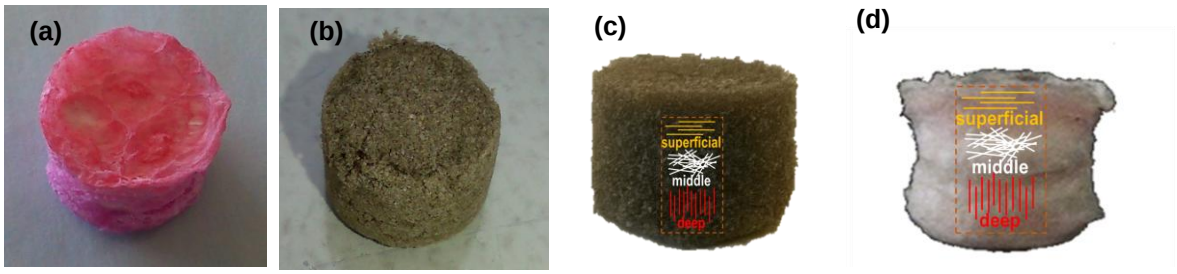


Figure 14: Final aspect of the scaffolds after being freeze-dried. (a) Collagen Scaffold; (b) GO-Chitosan-Collagen Scaffold and (c) Final aspect of a GO-Chit-Col scaffold with the expected fibre alignment of each zone.

2.4. PCL solutions characterization

2.4.1. Viscosity measurements

The viscosity of both the 15% and 12% PCL solution was measured with a rotational Malvern Kinexus Lab rheometer, at 25ºC with a minimum sheer stress of 0.1 s^{-1} and a maximum sheer stress of 100 s^{-1}. The tests were performed by Tiago Galvão, PhD from CICECO - Center for Research in Ceramics and Composite Materials, University of Aveiro.

2.5. Scaffold Characterization

2.5.1. ATR-FTIR

Fourier transform infrared spectroscopy (FTIR) is a very common spectroscopy technique based on the interaction between the infrared radiations (IR) and the material.[42] An important modality of FTIR, which is commonly used in surface composition characterization, is called Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR). Basically, this technique involves a close contact between the sample and an internal reflection element (e.g. zinc selenide and germanium crystals) that possess a high refractive index able to reflect the IR beam internally and then create an evanesce wave capable of penetrating in the immediate environment above the crystal. Some energy of the evanescence wave is then absorbed by the sample and then becomes a signal that later gets by Fourier Transform to a defined spectrum. The main advantage of this technique, relatively to FTIR, is that it doesn't need a previous preparation of the sample.[43]

The chemical analysis of the PCL electrospun fibres, the freeze-dried GO-Chit-Col hydrogel and a freeze-dried Col sample was performed ATR-FTIR (Bruker Tensor 27 FT-IR spectrometer - Bruker Corporation, Massachusetts, USA). The spectra were recorded between 4000 and 400 cm^{-1}, with a resolution of 4 cm^{-1} and 256 scans.

2.5.2. Scaffold Dimensions

After electrospinning, the dimensions of each PCL electrospun layer were measured after lyophilisation, using a caliber. The final assembled Col and GO-Chit-Col scaffolds were also measured, using the same approach. Triplicate measurements were carried out for every sample.

2.5.3. Scanning Electron Microscopy (SEM)

Scanning electron microscopy is a type of a morphological analysis technique. The formation of the image resulting from this analysis is dependent on the acquisition of signals produced by the interaction between the electron beam and the sample. These interactions can be divided into two categories: elastic and inelastic.[44]

The elastic scattering results from the deflection of the incident electron from the atomic nucleus of the sample or the outer layer of similar energy electrons.

The inelastic scattering occurs due to various interactions between the incident electrons and the electrons and / or the sample atoms. This results in the transference of energy to the atom from a primary beam of electrons. The excitation of electrons from the sample during the ionization of atoms results in secondary electrons, which conventionally are defined by having a lower energy of 50 eV and can provide information on the analysis of the sample image. [45]

The morphology of the electrospun PCL layers and the Col and GO-Chit-Col scaffolds was observed through a scanning electron microscope (Hitachi S-4100 Model – Hitachi High-Technologies Corporation, Germany).

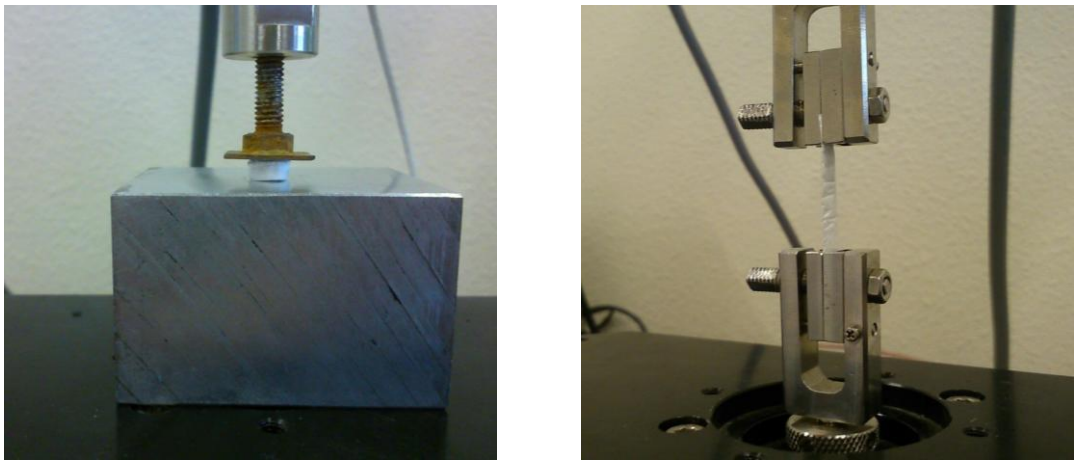
The samples subjected to this test were prepared in advance by coating them with a thin film of carbon (Emitech K950 equipment), in order to increase their conductivity.

The fiber sizes of the electrospun PCL layers were, later on, measured using the micrographs obtained after SEM analysis.

2.5.4. Mechanical Testing

The mechanical properties of the PCL electrospun layers and the Col and GO-Chit-Col scaffolds were determined using a Shimadzu MMT-101N (Shimadzu Scientific Instruments, Kyoto, Japan) with a load cell of 100 N. For the testing of the bottom and middle zones, compression tests were performed (Figure 16 (a)) whereas the PCL electrospun sheet was analyzed via tensile tests (Figure 16 (b)). In both cases the samples were compressed/stretched up to their maximum limit at a rate of 1 mm/min.

The compressive and tensile moduli of the samples were calculated by the analysis of the stress–strain curves obtained, specifically from the slope at low strain (0–15%). Triplicate measurements were carried out for every sample.



(a) **(b)**

Figure 15: Overall look of the mechanical tests. (a) Compressive; (b) Tensile

Chapter III

3. Results and discussion

3.1. PCL Solution Characterization

3.1.1. Viscosity Measurements

The results of the viscosity measurements are presented in the chart below.

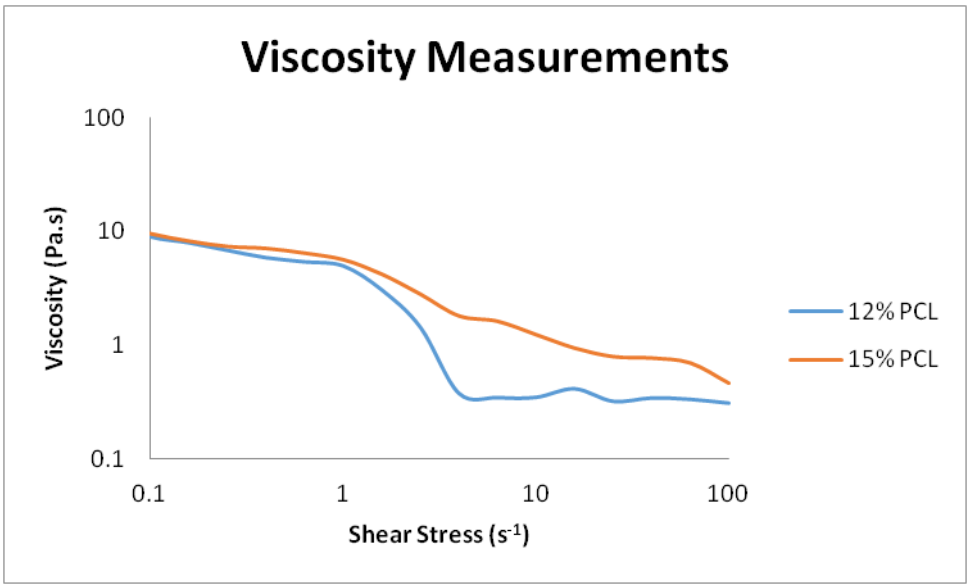


Figure 16: Graphic representation of the viscosity measurements of both PCL solutions (12 and 15%).

By the analysis of the curves, it is clear that for a higher percentage of PCL, the viscosity is also higher, as it was expected. [46]

Therefore, these two solutions are used for the development of two different zones of the scaffold, taking into consideration the changes made to, not only the set-up but also the electrospinning parameters used for both techniques.

The viscosity measurements were made only to ensure that the solution used for the wet electrospinning was less viscous than the one used for the dry electrospinning, due to the use of a smaller working distance. [30] This would avoid the appearance of beaded fibers. This measurement was of need because of the high volatility of the solvents used.

3.2. Scaffold Characterization

3.2.1. ATR-FTIR Analysis

As expected [47], the ATR-FTIR spectra of the PCL electrospun fibres presented in figure 17 corresponds to the spectra of standard PCL. Indeed, it is possible to observe an absorbance peak related with the asymmetric CH_2 stretching located at 2940 cm^{-1}, an intense absorbance peak associated with the carbonyl (C=O) stretching at 1724 cm^{-1} and it is also possible to see at 1240 cm^{-1} and 1170 cm^{-1}, the absorbance peaks related with the asymmetric and symmetric ether (C-O-C) stretching, respectively.

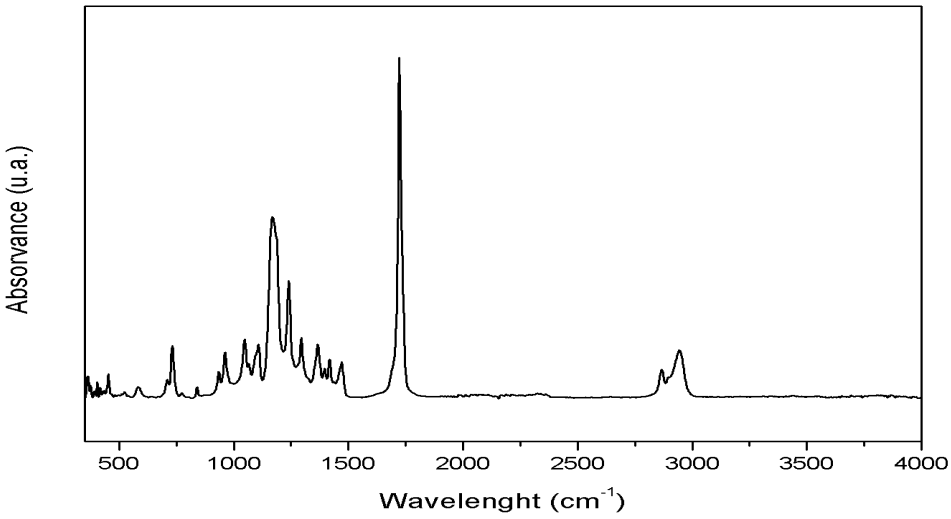


Figure 17: ATR-FTIR spectrum of the PCL electrospun fibres**.**

As it is possible to see from Figure 18, the ATR-FTIR spectrum of the GO-Chit-Col freeze-dried hydrogel shows characteristic bands of GO [48] Chit [49], and Col [50], presenting similarities with the ATR-FTIR spectrum of the GO-Col foam reported by Girão et al. In fact, the GO oxygen functional groups absorbance bands are located at 3350 cm^{-1} and 1049 cm^{-1} and related to the hydroxyl (OH) and alkoxy (C-O) functionalities. In addition to this, it is also possible to observe an absorbance peak located at 1630 cm^{-1}, which is not only related with the stretching vibrations of the C=C and the C=O groups of GO, but also with the C=O stretching of the amide I located both in the Col and Chit chains. The Col and Chit presence is also noticeable by that the high intensity and width of the absorption band located at 3350 cm^{-1}, which may be due to the combination of the OH groups from GO and the amine (N-H) stretch of both polymer chains. [51][52]

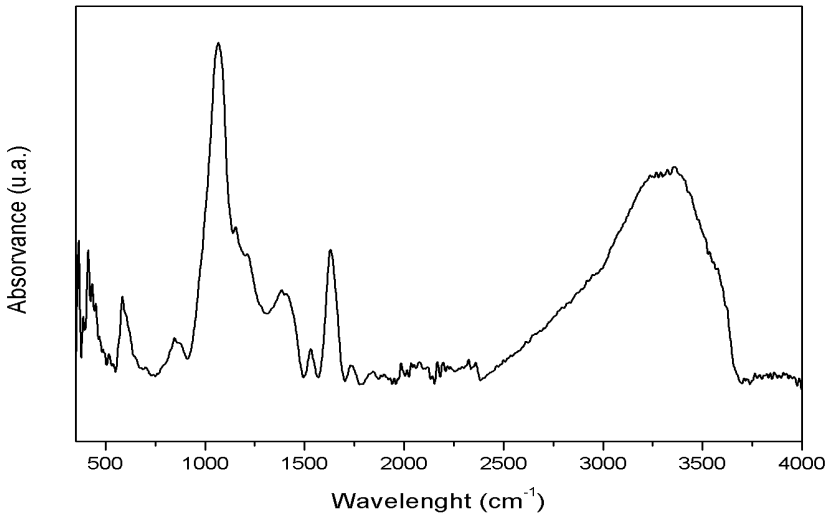


Figure 18: ATR-FTIR spectrum of the GO-Chit-Col freeze-dried hydrogel.

Figure 19 presents the ATR-FTIR spectrum of a freeze-dried Col sample.[52] As expected, the N-H stretching vibration is visible at 3440 cm^{-1}. The other characteristic peaks are located at 1660 and 1290 cm^{-1} are related with the stretching of the Amide I in the Col chain and the N-H bending coupled with the C-N stretching of the Amide III located in the Col structure, respectively. [52]

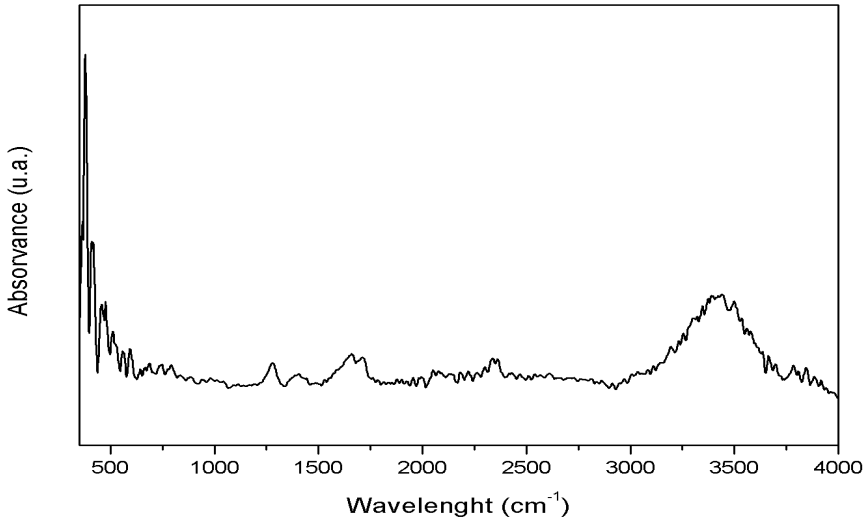


Figure 19: ATR-FTIR spectrum of the Col freeze-dried sample.

3.2.2. Scaffold dimensions

The tables below describe the measurements of each zone of the scaffold, as it was described in section 3.5.2 of Chapter II. Each sample was named by the initial of the zone it is referred to. Therefore, S represents the superficial zone, M de middle zone and D the deep zone. By the analysis of the values described in Table 1, it is possible to say that the fabrication processes of the PCL electrospun zones are reproducible, considering the standard deviation obtained for their diameter (d) and height (h).

Indeed, the superficial zone presents the expected diameter and a height that corresponds to the thickness of the PCL electrospun sheet.

Regarding the middle zone, both the diameter and height measured present higher and lower dimensions than the original mold (5 mm diameter x 3 mm height) respectively. This is probably due to the lyophilization process that removes the ethanol from the PCL electrospun layer and consequently causes its flattening.

The deep zone also presents not only a higher diameter than expected but also a higher height than the original mold (5 mm diameter x 3 mm height). This may be due to the removal of the PCL electrospun layer from the mold that can originate the detachment of the three PCL electrospun sheets used for the deep zone fabrication. Additionally, the diameter of this zone is slightly higher than expected (3 mm) because it was manually cut.

Taking into account these results, the overall 3D electrospun structure presents a total height of approximately 5.72 mm, which is compatible with the original goal (a 5 mm high structure).

Table 1: Dimensions of the PCL electrospun zones.

Sample	*d(mm)*	*h(mm)*
S	5.31±0.25	0.120
M	6.83±0.15	2.10±0.45
D	7.43±0.59	3.50±0.60

The table 2 presents the measurements of the dimensions of the Col and GO-Chit-Col scaffolds, confirming that in both cases the lyophilization process did not affect their expected diameter (1 cm). Concerning the height of the scaffolds, the GO-Chit-Col scaffold maintained the dimensions of the mold (1 cm) whereas the Col scaffold suffered shrinking due to water removal during the lyophilization process. This is a plausible result

since the Col hydrogel contains a higher amount of liquid compared to the GO-Chit-Col hydrogel.

Table 2: Dimensions of the Col and GO-Chit-Col scaffolds.

Scaffold	*d(mm)*	*h(mm)*
Col	8.95±0.45	6.20±0.57
GO-Chit-Col	1.00±0.10	1.00±0.12

3.2.3. SEM analysis

By the analysis of figures 20 and 21, it is possible to see that the PCL fibres have the alignment expected for these two zones, more specifically aligned in the top zone, as the collagen fibres are aligned in the superficial zone of cartilage tissue, and randomly aligned in the middle zone. As to the deep zone (Figure 22), because the electrospinning conditions used for the development of this zone were the same as the ones used for the superficial zone and considering the methodology followed (Chapter II, section 2.3.3.), it can be assumed that, in this zone, the fibres are vertically aligned. It is also noticeable that theoretically, there is enough space inside the PCL electrospun spiraled structure, to ensure good cell adhesion, proliferation and migration. [11]

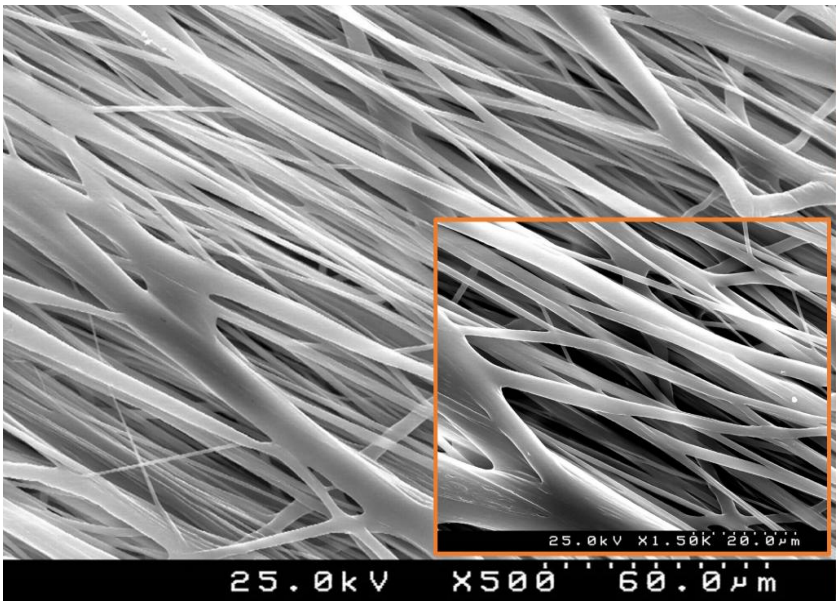


Figure 20 Migrographs of the electrospun PCL zone: Top zone;

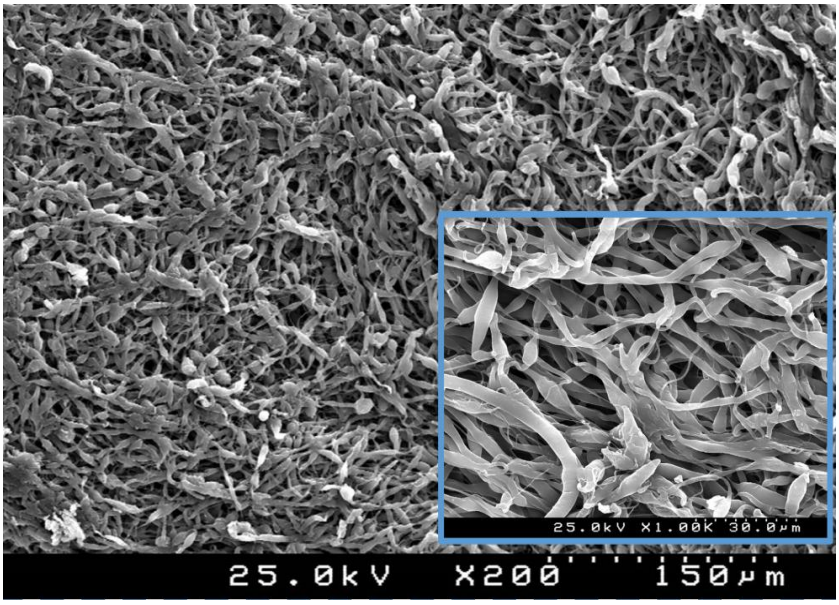


Figure 21 Micrographs of the electrospun PCL layer: Middle zone;

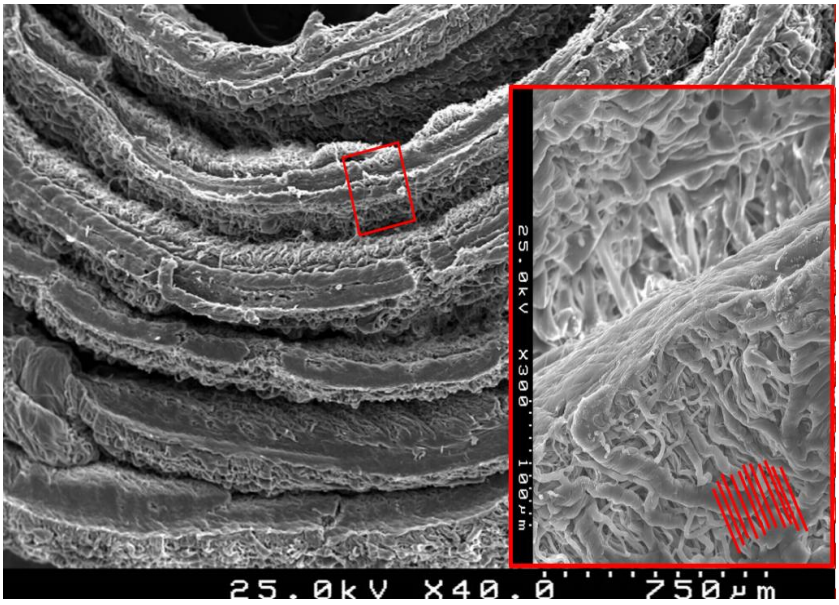


Figure 22 Migrographs of the electrospun PCL layer:: Deep zone

In fact, each cartilaginous zone shows analogous architecture and topography relatively to its native counterpart due to the accurate modulation of the fibre orientation via electrospinning.

Using Image J, the diameter of the fibres from each electrospun zone was also measured (Table 3).

Table 3: Fibre size measurements for each zone of the produced scaffold.

Zone	*Fibre Size(µm)*
S	[0.47 - 10.17]
M	[0.17 - 10.64]
D	[0.47 - 10.17]

By the analysis of the table 3, it is noticeable that there is a very wide range of fibre sizes in each PCL electrospun zone. This may be due to the ranges of electrospinning conditions that were used in order to avoid the formation of clots in the electrospinning needle that would eventually lead to the appearance of beads. [46] The presence of higher diameter fibres (around 10 µm), although not in large number, can be explained by considering the merge of the small diameter fibres probably due to an inefficient solvent evaporation, an insufficient working distance or not optimal temperature and humidity conditions during the electrospinning process. [46]

One can also assume that there is no possible way of comparing both fibre sizes obtained by using the different electrospinning processes (dry and wet method) due to the disparity of conditions that were imposed in both mechanisms.

Figures 23 and 24 show the top views of the Col scaffold and GO-Chit-Col scaffold, respectively. By the analysis of the micrographs, it is noticeable that the GO-Chit-Col scaffold is considerably more porous than the Col scaffold because of the electrostatic interactions presented during GO-Chit-Col hydrogel synthesis between the negatively charged GO sheets and the positively charged Col and Chit particles. In fact, the polymers work as physical crosslinkers between the GO sheets in acidic medium, leading to the hydrogel formation that originates stable microporous foam.[25][53][54]

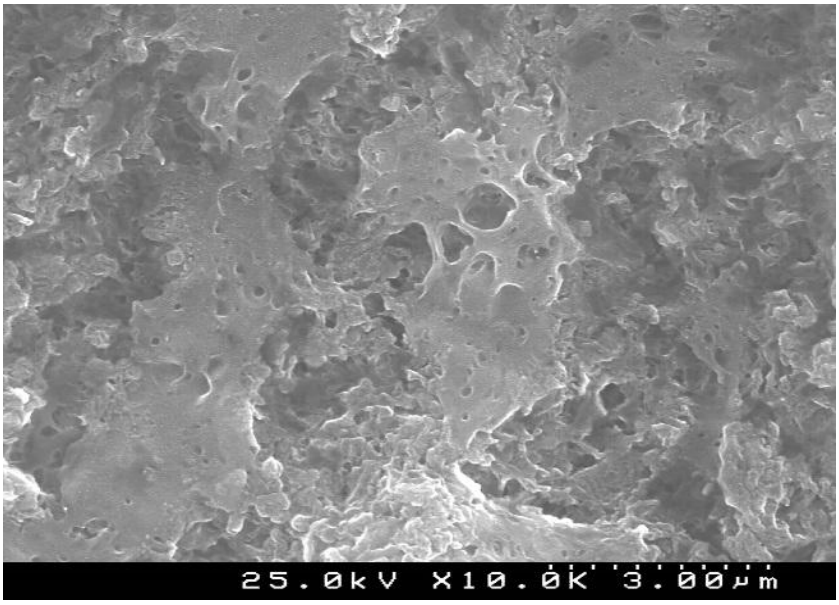


Figure 23: SEM micrograph of the top view of the Col scaffold.

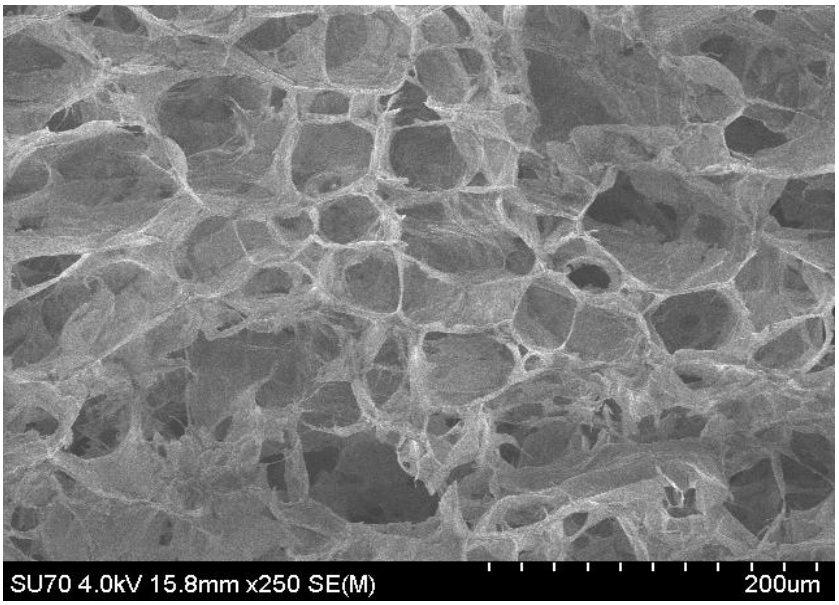


Figure 24: SEM micrograph of the top view of the GO-Chit-Col scaffold.

Figures 25 and 26 present two transversal cuts of the Col scaffold, where it is possible to observe that neither the assembly of the middle zone (Figure 25) and deep zone (Figure 26) inside the hydrogel nor the lyophilization process have affected the integrity of the PCL electrospun fibres in use.

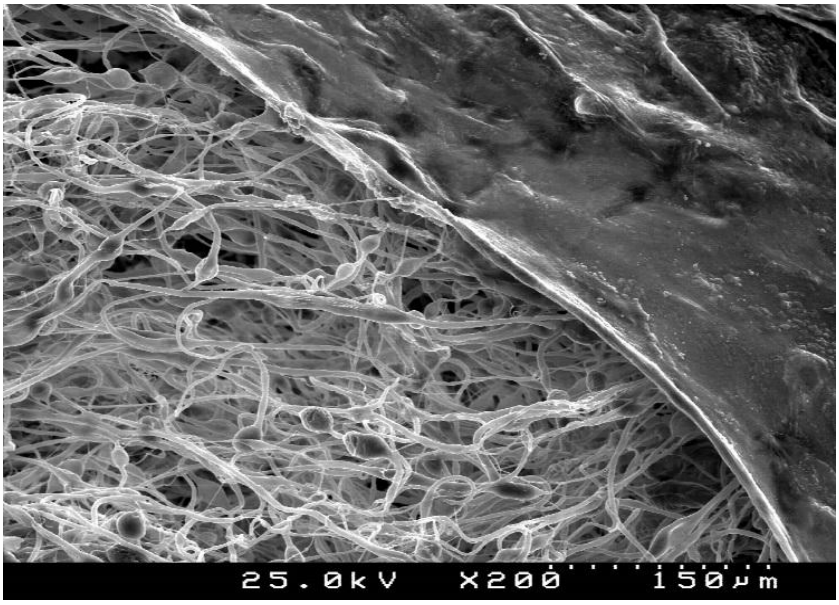


Figure 25: Transversal cut of the middle zone of the Col scaffold.

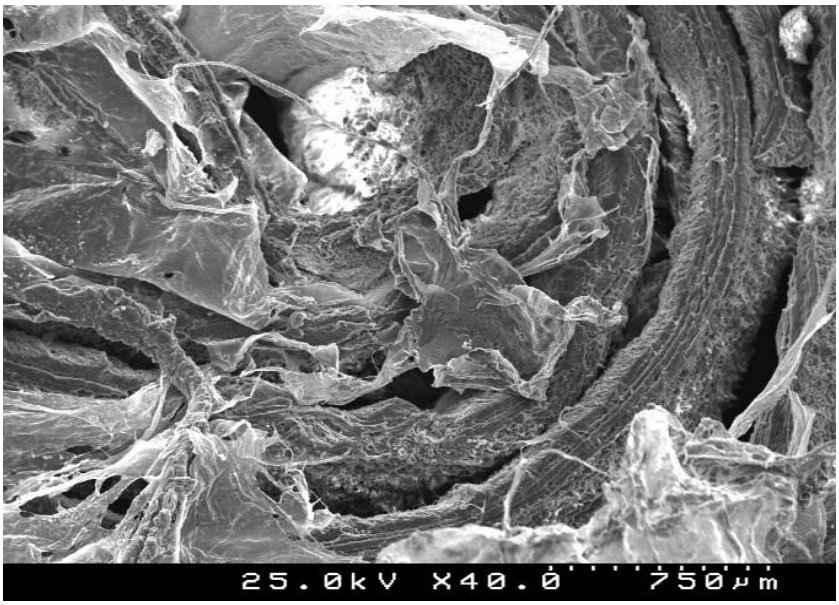


Figure 26: Transversal cut of the bottom zone of the Col scaffold.

3.3.3. Mechanical Properties

Using Equations 1 and 2, adding to the measurements described in section 3.3.3, the Strain and Stress which the scaffolds were under, were properly obtained.

$$e = \frac{\Delta L}{L_0} \qquad \textbf{(1)}$$

In which ε represents the strain, ΔL represents the displacement variation suffered by the scaffold, and L_0 its initial height.

$$\sigma(Pa) = \frac{F\ (N)}{A(m^2)} \quad \textbf{(2)}$$

Where σ is representative of the strain the samples were under, F represents the Force and A the Area in which the Force values were applied.

Table 4 summarizes the results obtained during the mechanical testing of the PCL electrospun layers. As it is possible to observe, the deep and middle zones show a compressive moduli appropriate for protocols involving cartilage tissue engineering approaches. [1] [2] The huge difference between the values measured is mainly related with the density of the electrospun fibers, which is higher for the deep zone. Indeed, the middle zone presents a much noticeable porous network (Figure 22) compared to the deep zone, which is coherent with other works involving wet electrospinning procedures.[55]

The tensile tests regarding the superficial zone showed a tensile modulus of 1MP [56][57], a value consistent with other PCL electrospun meshes reported in literature. However, it is possible that the calculated tensile modulus could be lower than the real value since the claws used during the tensile tests (Figure 16b) did not present the appropriate size to fix the PCL electrospun layer in an optimized manner.

Table 4: Middle zone compressive moduli of each sample.

Zone	*Compressive Modulus (KPa)*	*Tensile Modulus(MPa)*
S		1.1 ± 0.1
M	22.6 ±6.7	
D	192.4±8.2	

Regarding both the Col and GO-Chit-Col scaffold, it is possible to see from their stress-strain curves (Figures 27 and 28) that none of the scaffolds fracture at the stress levels applied. The compressive modulus of the Col scaffold 31.0 ± 7.0 kPa is similar to

the compression modulus calculated for the middle zone, revealing that the Col porous structure that surrounds the 3D PCL fibrous structure did not significantly affect the overall mechanical properties of the scaffold. On the other hand, the compressive modulus of the GO-Chit-Col scaffold exposes that during the first 15% of strain [5], only the porous network that surrounds the 3D PCL fibrous structure is affected. This hypothesis is reinforced by the similarity between the compressive moduli of this scaffold (14.5±2.0 kPa) and the GO-Col foams reported by Girao et al. (15.75 ± 0.64 kPa).

The difference between the compressive moduli of the fabricated scaffolds is mainly related to the highly porous structure obtained for the GO-Cs-Col scaffold during the freeze-drying process, leading to the placement of the 3D PCL fibrous structure (similar for both scaffolds) inside a bigger porous network and therefore to the mitigation of the influence of the fibrous structure for the first 15% strain.[5]

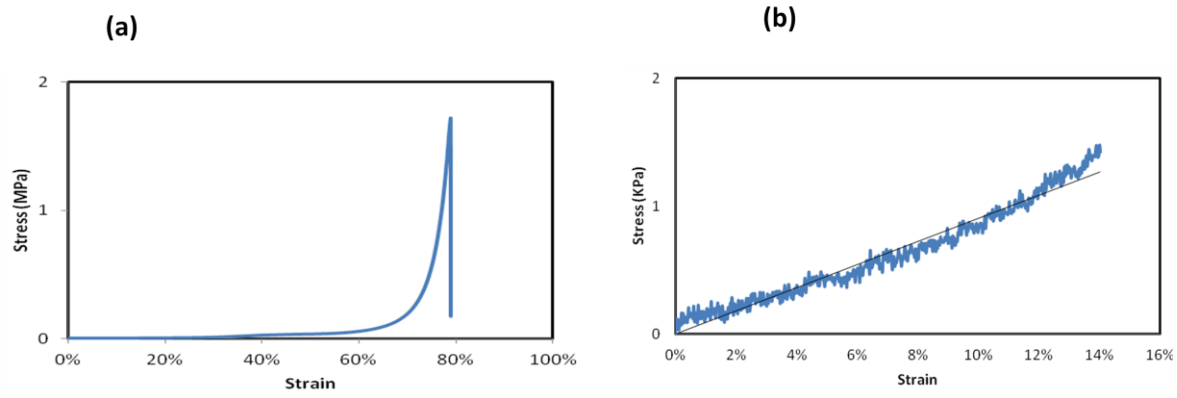


Figure 27: Graphic representation of the Col-scaffold mechanical tests. (a) Overall aspect of the stress-strain curve; (b) curve of the 15% strain.

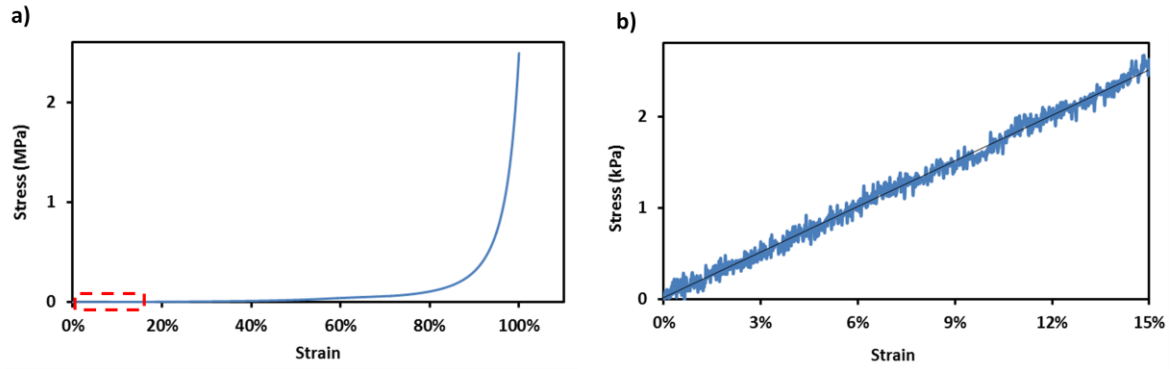


Figure 28: Graphic representation of the GO-Chit-Col -scaffold mechanical tests. (a) Overall aspect of the stress-strain curve; (b) curve of the 15% strain.

Chapter IV

4. Conclusions and future work

There are many challenges in tissue engineering involving every aspect regarding the creation of a structure that would successfully mimic the environment in which it would later on be implanted. From the materials of choice, to the techniques in use, a lot of barriers need to be overcome.

Therefore, towards developing an innovating way of regenerating cartilaginous tissue was to electrospun scaffolds of PCL with depth dependent variations in the fibrilar size and orientation, in order to mimic cartilage's natural topographical and architectural characteristics, in terms of collagen fiber alignment and orientation. These scaffolds were successfully produced in order to provide not only those topographical and architectural features, but also to provide guidance and support for cell migration and proliferation. The scaffolds were designed and fabricated to mimic the natural extracellular cell matrix of cartilaginous tissue.

The alignment and deposition of the electrospun fibres were managed by varying the electrospinning conditions in use, in which the type of collector in use played a crucial role.

After producing and characterizing both the Col and GO-Chit-Col scaffolds, it is noticeable that each PCL elecrospun zone shows analogous architecture and topography relatively to its native counterpart due to the accurate modulation of the fibre orientation via electrospinning.

Even though the final size of the scaffold does not match the ideal thickness for cartilage tissue engineering purposes, this is only a prove of concept, which means the scaffolds were created in order to prove that there was a possibility of producing a scaffold with the morphological organization of the collagen fibres on the cartilaginous tissue.

Although the mechanical properties of the scaffold are suitable for protocols involving cartilage tissue engineering approaches, the assembly process needs some design and fabrication upgrades.

Despite all the work, there are always things that can be done in an attempt to improve the system under development.

Future work will include the study of cell migration through the created Col and GO-Chit-Col scaffolds in order to guarantee that the final cellular distribution is similar to the condrocytes' distribution throughout the natural tissue;

Also, it is important to test how the mechanical properties adjust taking into account the thickness of each electrospun PCL zone, in order to quantify the involvement of each zone in the response to mechanical stimulus.

Finally, static and dynamic tests (via bioreactor) are also an important validation method, using cell culture protocols.

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Annexes

The quality and innovation of the scientific work reported in this thesis was already been recognized by the scientific community, as proof the recent acceptances for oral and poster presentation in both national and international congresses:

Annex 1: Poster titled “Physiological Collagen Architecture in Engineered Tissue Cartilage Through a Combined Approach of Mechanical Stimulus and Anisotropic Fibrous Scaffolds” presented at the 5th International Conference on Integrity -Reliability – Failure, July 2016, Porto, Portugal.
List of authors: André F. Girão, **Ana J. Nóbrega**, Paula A. A. P. Marques and António Completo

Annex 2: Oral presentation titled “A biomimetic three-dimensional anisotropic nanofibrous polycaprolactone-collagen scaffold for cartilage tissue engineering” presented at the 11th International Conference on Surfaces, Coatings and Nanostructured Materials (NANOSMAT), September 2016, Aveiro, Portugal.
2nd place at the Young Scientist Lecture Competition.
List of authors: **Ana J. Nóbrega**, André F. Girão, Paula A. A. P. Marques and António Completo.

Annex 3: Accepted oral presentation titled “DEVELOPMENT OF A PHYSIOLOGICAL COLLAGEN ARCHITECTURE THROUGH ANISOTROPIC NANOFIBROUS POLYCAPROLACTONE-COLLAGEN SCAFFOLD FOR CARTILAGE TISSUE ENGINEERING” that will be presented at the 7º CONGRESSO NACIONAL DE BIOMECÂNICA, February 2017, Guimarães, Portugal.
List of authors: **Ana J. Nóbrega**, André F. Girão, Paula A. A. P. Marques and António Completo.

Annex 4: Submitted abstract titled “A graphene-oxide-collagen scaffold as a versatile three-dimensional biomimetic microenvironment for tissue engineering applications” that will be presented at the XVIII Congresso da Sociedade Portuguesa dos Materiais, April 2017, Aveiro, Portugal.
List of authors: André F. Girão, Gil Gonçalves, **Ana J. Nóbrega**, Sofia S. Almeida, António Completo and Paula A.A.P. Marques

Annex 1

PHYSIOLOGICAL COLLAGEN ARCHITECTURE IN ENGINEERED TISSUE CARTILAGE THROUGH A COMBINED APPROACH OF MECHANICAL STIMULUS AND ANISOTROPIC FIBROUS SAFFOLDS

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ABSTRACT

This work consists on improvement of the functionality of tissue engineered cartilage through a combined approach of mechanical stimulus with anisotropic fibrous scaffolds provided on a bioreactor culture environment, in order to bring the structural organization of engineered cartilage close to the native tissue, reducing the risk of failure of this promising method for osteoarthritis treatment.

Keywords: biomechanics, mechanical stimulus, anisotropic fibrous scaffolds

INTRODUCTION

The major challenge associated with tissue engineered cartilage is the difficulty to approximate the mechanical properties of the engineered tissues to the native ones (Johnstone et al 2013). Various experimental studies in bioreactors had concluded that the mechanical properties of engineered cartilage can be improved by appropriate mechanical stimulation (Kock et al 2012). These indications are promising, but the properties of these engineered cartilages remain inferior to native. The major shortcoming of tissue-engineered cartilage is believed to be the lack of collagen content and consequently its poor tensile properties. Collagen reaches only 15-35% of the native content after 5–12 weeks (Kock et al, 2012). Another shortcoming is that tissue-engineered cartilage does not possess native zonal variations. The importance of the arcade-like collagen structure for the load-bearing properties of native cartilage is well emphasized in literature (Danisovic et al, 2012). However, despite extensive cartilage tissue engineering research, few studies have assessed the importance of collagen fibril depth-orientation on the mechanical properties of engineered cartilage. Culture conditions

that have impact on collagen synthesis and fibril organization in-vitro include scaffold properties (McCullen et al, 2012) and mechanical stimulation (Bandeiras et al, 2014; Bandeiras et al, 2015). Future research should particularly focus on approaches to increase collagen content and fibril organization. For tissue engineered cartilage to be mechanically functional, some investigators, believe that depth arcade-like collagen architecture should be reproduced to some extent in engineered cartilage. But how can this be best achieved? Some investigators hypothesize that applying depth-varying mechanical cues would stimulate extracellular matrix synthesis/organization depth dependently (Kock et al 2012). Others, suggested the use of anisotropic fibrous scaffolds, in order to provide a template to organize the newly deposited matrix (McCullen et 2012).

RESULTS AND CONCLUSIONS

This work explores different process parameters, related with electrospun scaffolds, like fiber size, spacing and orientation to produce scaffolds with different depth-dependent structural organizations through a nanofiber Electrospinning machine (McCullen et al, 2012). By varying the fiber size, the resultant pore size and mechanical strength can be varied over a wide range. Fiber orientation can be varied by modifying collector geometry or rotating collector speed, yielding fibrous scaffolds that vary from randomly oriented scaffolds to highly aligned networks with concomitant variations in tensile strength (McCullen et al, 2012). A technique to incorporate multiple electrospun layers with distinct fiber sizes and orientations is that of sequential electrospinning onto the same collector under varying operating conditions. Additionally, by exploring the mechanical stimulus parameters like load type (compression, traction, shear, hydrostatic pressure or combined), load magnitude, applied frequency and loading period during the culture phase, in an active bioreactor previously developed, it will be possible to generate different strain distribution along scaffold depth which may enhances collagen content and a depth-varying collagen orientation (Bandeiras et al, 2014). A promise loading regime involving sliding of the indenter with lateral compression may stimulate the formation of an arcade-like collagen architecture.

ACKNOWLEDGMENTS

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Annex 2

A biomimetic three-dimensional anisotropic nanofibrous polycaprolactone-collagen scaffold for cartilage tissue engineering

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The major challenge associated with tissue engineered cartilage is the difficulty to approximate the properties of the engineered tissues to the native ones due to cartilage's depth-dependent microstructural organization, which is divided into three functionally complementary nanofibrous zones responsible for balancing optimal mechanical properties with an enhanced biochemical cell response [1,2].
Therefore, in this study, we manufactured a three-dimensional (3D) anisotropic nanofibrous polycaprolactone (PCL) scaffold with depth-dependent variations in fibre size and orientation able to mimic the natural collagen (Col) fibrous network present on the cartilage microenvironment. Indeed, the distinctive features of each cartilaginous zone were replicated by different electrospinning set ups and then assembled within a Col hydrogel, leading to an innovative 3D structure capable of combining an accurate organizational and morphological fibre distribution with a biocompatible porous system. The PCL-Col scaffold was then characterized via SEM analysis and its mechanical properties were evaluated via static and dynamic compression tests under physiological conditions.
Based on the accomplished results, we propose that the described cartilage-like PCL-Col scaffold could be a viable candidate for biological studies using chondrocytes towards new strategies for cartilage tissue engineering.

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Annex 3

Development of a physiological collagen architecture through anisotropic nanofibrous polycaprolactone-collagen scaffold for cartilage tissue engineering

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KEYWORDS: Cartilage Tissue Engineering, Biomaterials, Electrospinning, Anisotropic Nanofibrous Scaffold

SUMMARY: *In this study, we propose an innovative 3D electrospun scaffold with distinct biomimetic zones capable of both simulating the collagen fibrous architecture of the native cartilage and presenting mechanical features that show potential to be compatible with both static and dynamic cell culture protocols.*

1 Introduction

The main goal of cartilage tissue engineering (TE) is the *in vitro* recreation of the depth dependent nanostructural organization of the fibrous collagen network that comprises the cartilage natural extracellular matrix. In fact, native cartilage is anatomically and functionally divided into three nanofibrous zones with distinct mechanical properties due to fiber size and orientation, progressing from perpendicular to the subchondral bone surface in the deepest zone, to random in the middle zone and to parallel in the superficial region. Though the promising results of both fibrous and porous scaffolds used to overcome this challenge during the past few years [1], none of the followed methodologies is currently able to guarantee an optimal balance between biological features, mechanical properties and suitable topographic cues. Additionally, recent studies have indicated the necessity to include mechanical stimulation via a bioreactor in the scaffolding strategy in order to enhance the modulation of the chondrocyte behavior *in vitro* [2].

Taking all this into account, we have developed a newly 3D scaffold capable of not only simulating each cartilaginous zone with great morphological accuracy, but also providing a biocompatible porous network suitable for both static and dynamic cell culture protocols.

2 Methodology

To recreate each cartilaginous zone, different electrospinning set ups, including a rotating mandrel and an ethanol-water bath as collectors, were used to fabricate Polycaprolactone (PCL) nanofibres with controlled size and alignment. In a second stage, the fibrous biomimetic zones were assembled within a collagen hydrogel that after a lyophilisation process was able to physically support the fibres inside a heterogeneous porous network.
The individual parts of the 3D PCL-collagen scaffold were characterized via SEM analysis and their mechanical properties were evaluated via static and dynamic (via a bioreactor) compressive and tensile tests.

3 Results and Discussion

As it is illustrated in Fig. 1, the design and fabrication methods adopted were able to successfully incorporate three biomimetic layers of PCL fibres within a collagen porous network.

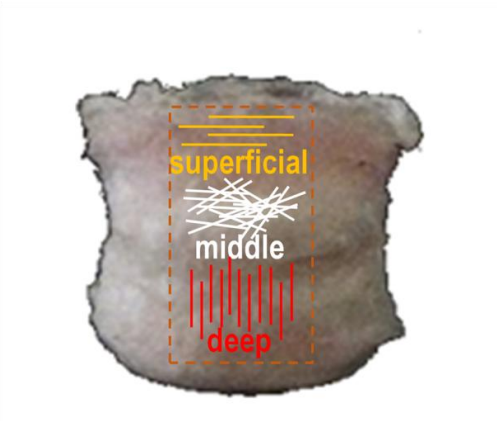

Fig. 29. 3D PCL-collagen scaffold: scheme of the PCL biomimetic zones assembled inside the collagen porous network

SEM analysis (Figs. 2, 3 and 4) has confirmed that each PCL fibrous layer shows analogous architecture and topography relatively to its native counterpart due to the precise modulation of the fibre orientation via electrospinning. Relatively to the mechanical properties and size of the electrospun fibres (Tab. 1), the three zones presented Young moduli and diameters suitable for protocols involving cartilage TE strategies [3]. These preliminary results suggest that the presented scaffold can offer an enhanced cellular microenvironment capable of promoting cartilage regeneration.

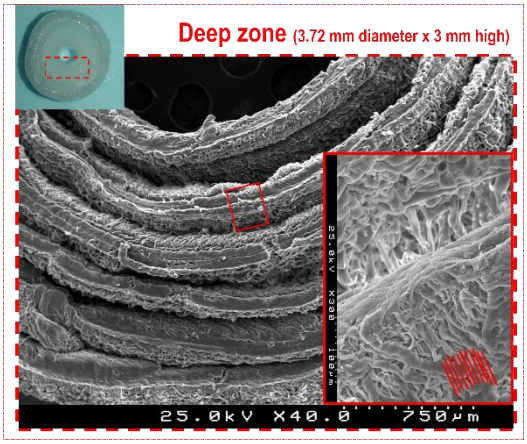

Fig. 30. Deep zone: SEM analysis.

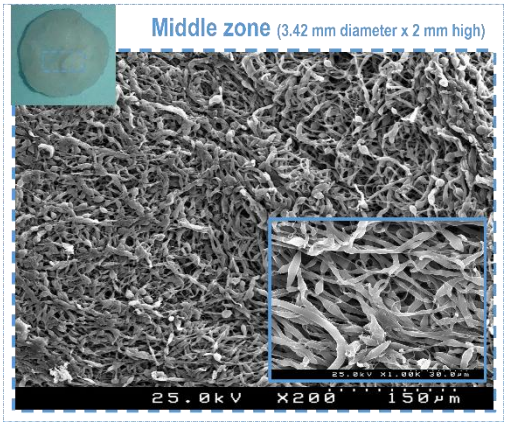

Fig. 31. Middle zone: SEM analysis.

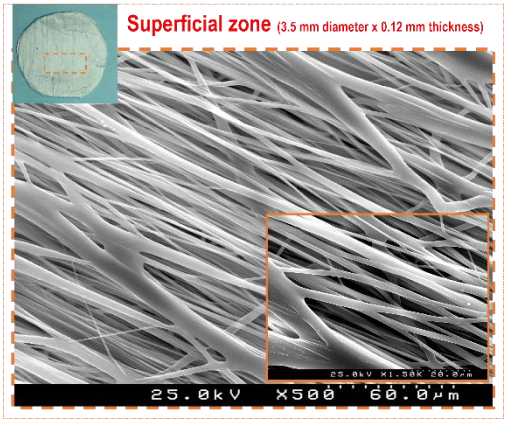

Fig. 32. Superficial zone: SEM analysis.

Tab. 1 Properties of the electrospun fibres

Zone	Diameter (μm)	Young M. (kPa)
Deep	2.8 ± 1.8	192.4 ± 8.2
Middle	4.8 ± 2.6	22.6 ± 6.7
Superficial	2.8 ± 1.8	1100 ± 100

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Annex 4

A graphene-oxide-collagen scaffold as a versatile three-dimensional biomimetic microenvironment for tissue engineering applications

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During the past few years, graphene and its hybrids have become increasingly popular biomaterials for tissue engineering (TE) applications due to not only their excellent biochemical, electrical and mechanical properties, but also because of their potential to be combined with other materials in order to fabricate biocompatible composites with superior behavior. In this regard, graphene oxide (GO) has emerged as front runner to act like a functional building block of complex 3D cellular microenvironments since the oxygen functional groups located onto the surface of its sheets can be complementary used to directly enhance cell response and to be combined with specific biomolecules and polymers.[1,2]

Thus, in this work we successfully explore the electrostatic interactions between the negatively charged GO sheets and the positively charged collagen (Col) particles in order to fabricate a self-assembled 3D porous scaffold capable of guarantee suitable biochemical and biomechanical features for a wide range of TE strategies, involving both static and dynamic cell culture protocols.[3] Indeed, the chemical, mechanical and morphological properties of the GO-Col scaffold were minutely analyzed and its capability to potentiate an enhanced cellular microenvironment was confirmed by preliminary biocompatibility tests using Rat Schwann cells. The versatility of the scaffold was also revealed through their compression-recovery features since independently of the degree of deformation applied (1%, 3% and 7%) via a bioreactor apparatus, its integrity was not affected. Additionally, these promising results boosted the adaptation of this GO-Col scaffold into the cartilage TE applications by innovatively incorporating inside the porous network an anisotropic electrospun scaffold able to mimic with great morphological accuracy the cartilaginous natural fibrous network.

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